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# Structuring solid/oil/water emulsions to deliver water-soluble bioactive food ingredients

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To the Graduate Council:

I am submitting herewith a dissertation written by Yun Zhang entitled "Structuring solid/oil/water emulsions to deliver water-soluble bioactive food ingredients." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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# Structuring solid/oil/water emulsions to deliver water-soluble bioactive food ingredients

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# ABSTRACT

Numerous delivery systems are studied for lipophilic bioactive compounds, but much work is needed for bioactive food ingredients soluble or dispersible in water. The overall hypothesis of this dissertation is that structured solid/oil/water (S/O/W) emulsions can be used to encapsulate water-soluble/dispersible ingredients to retain their release during processing and storage and control their release during simulated digestions. The encapsulation principle is to prepare spray-dried powder of water-soluble/dispersible compounds as the solid core that is coated by an oil layer emulsified by food biopolymers. The oil layer isolates the compounds from the continuous aqueous phase in emulsions to prevent the release, and the digestion of food biopolymers enables the controlled release. Glutamine was first studied as a model water-soluble compound in Chapter 2, and the S/O/W emulsion droplets with sequential protein (whey protein isolate or sodium caseinate) and citrus pectin interfacial layers minimized the release of glutamine during storage and simulated gastric digestion but gradual and complete release during simulated intestinal digestion. The solid core was substituted for spray-dried probiotic *Lactobacillus salivarius* NRRL B-30514 in Chapter 3, and double-layered emulsions improved the bacteria viability during storage at 4 ℃, after heating, and *in vitro* digestion. In Chapter 4, sugar beet pectin, a natural protein-polysaccharide conjugate, was used to prepare S/O/W emulsions, and similar improvements of bacteria viability were observed. In Chapter 5, outlet temperature, drying media, and heat adaptation were optimized to improve the viability of *L.*

*salivarius* after spray drying and subsequent storage. Representative spray-dried *L. salivarius* samples were encapsulated in droplets emulsified with sugar beet pectin in Chapter 6. Treatments of spray-dried *L. salivarius* samples with a lower water activity and those with disaccharides enhanced the viability of bacteria during storage at 22ºC and 11% RH. Spray-dried lactase was studied in Chapter 7 as the last model compound. Encapsulation in S/O/W emulsions improved the preservation of lactase during thermal processing and storage to minimize lactose hydrolysis in milk, and enabled the release of lactase and hydrolysis of lactose during simulated gastric and intestinal digestions. Therefore, the studied S/O/W emulsions are significant to the production of numerous functional foods.

**Keywords**: glutamine, probiotics, lactase, encapsulation, storage, pasteurization, in vitro digestion.

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# Chapter 1 . Introduction and literature review

## 1.1 An overview of bioactive food ingredients

Food ingredients with physiological functions are known as bioactive food ingredients (Augustin & Hemar, 2009). In recent years, more and more consumers pay much attention on health benefits of foods beyond satisfying one's hunger and the taste (Patel & Velikov, 2011). Consequently, bioactive food ingredients with health promoting or disease preventing effect have been utilized in food industry to produce functional foods (Day, Seymour, Pitts, Konczak, & Lundin, 2009). In this section, common bioactive food ingredients applied to production of functional foods are reviewed.

### *1.1.1 Lipids*

Lipids are an important source of energy and many lipids are nutraceuticals. Generally, bioactive lipids include fatty acids, carotenoids, phytosterols and fat soluble vitamins (de Vos, Faas, Spasojevic, & Sikkema, 2010).

#### *1.1.1.1 Fatty acids*

Generally, bioactive fatty acids include Omega-3 fatty acids, short chain fatty acids (Saupe, Wissing, Lenk, Schmidt, & Müller, 2004) and conjugated fatty acids (D Julian McClements, Decker, & Weiss, 2007).

Omega-3 fatty acids are also known as Omega-3 polyunsaturated fatty acids, which have at least two double bonds and the first double bond starts from the third carbon counting from the methyl end (Ruxton, Reed, Simpson, & Millington, 2004). For example, docosahexaenoic acid (DHA, 22:6), eicosapentaenoic acid (EPA, 20:5) and α-linolenic acid. All these Omega-3 fatty

acids have been found to possess positive effect on cardiovascular function, mental health, and function of brain (Ruxton, Reed, Simpson, & Millington, 2004; Swanson, Block, & Mousa, 2012).

Short chain fatty acids are composed of 1-6 carbons and are produced by the fermentation of carbohydrates in the gastrointestinal tract of mammals (Rombeau, Kripke, & Settle, 1990). Acetic, propionic, and butyric acids are the three major short chain fatty acids that have been reported to prevent colonic disease (Scheppach, Bartram, & Richter, 1995).

Conjugated fatty acids are a mixture of positional and geometric isomers of polyunsaturated fatty acids; they have been drawn attention due to their potential effects on alleviating some diseases (Nagao & Yanagita, 2005). Conjugated linoleic acid has been reported to possess anti-cancer property due to the inhibition of cancer cell growth (Shultz, Chew, Seaman, & Luedecke, 1992). Besides, conjugated linoleic acid has anti-obesity property (Terpstra, 2004).

*1.1.1.2 Carotenoids*

Carotenoids are a group of lipophilic compounds that are produced by plants and microorganisms, and they are responsible for the orange, yellow and red colors of fruits and vegetables. β-carotene, α-carotene, lycopene, zeaxanthin and lutein are the predominant carotenoids (Rao & Rao, 2007).

Carotenoids are considered as bioactive food ingredients due to the ability to prevent cancer, cardiovascular diseases and other chronic diseases (Mayne, 1996; Paiva & Russell,

1999). For example, research demonstrated that β-carotein had effect on lung cancer (Van Poppel & Goldbohm, 1995). Consumption of lycopene-rich food could reduce the risk of esophageal cancer (Cook-Mozaffari, Azordegan, Day, Ressicaud, Sabai, & Aramesh, 1979). Zeaxanthin and lutein are carotenoids containing oxygen, and are found in retina and lens (D Julian McClements, Decker, & Weiss, 2007). These two carotenoids, as antioxidant ingredients, are able to quench the exited triplet states or singlet molecular oxygen to prevent ocular tissue from photo-oxidative damage (Stahl & Sies, 2005).

*1.1.1.3 Phytosterols*

Phytosterols are a group of sterols exclusively existing in plants, with similar structures and functions as cholesterols in animals (Ostlund Jr, 2002). β-sitosterol, campesterol and stigmasterol are the most common dietary phytosterols (Figure 1-1). Generally, best dietary sources of phytosterols include seeds, nuts, unrefined oils and legumes (Awad, Chan, Downie, & Fink, 2000; Weihrauch & Gardner, 1978). Phytosterols have been widely studied due to their properties of reducing cholesterol level and anticancer (Awad & Fink, 2000). Various studies concluded that phytosterols can prevent lung cancer (Mendilaharsu, De Stefani, Deneo-Pellegrini, Carzoglio, & Ronco, 1998), stomach cancer (De Stefani, Boffetta, Ronco, Brennan, Deneo-Pellegrini, Carzoglio, et al., 2000), and ovary and human breast cancer (Ju, Clausen, Allred, Almada, & Helferich, 2004; S. E. McCann, Freudenheim, Marshall, & Graham, 2003).

*1.1.1.4 Fat soluble vitamins*

Fat soluble vitamins A, D, E and K are kept in adipose tissue with considerable quantities (Lukaski, 2004). Fat soluble vitamins are considered as elemental nutrients for health and body functions (He, Lawrence, & Liu, 1992).

Vitamin A and its derivatives, includring retinaldehyde, retinol, and retinoic acid, play a significant role on health, vision, growth, and immune functions (Lukaski, 2004). Vitamin D is a family of secosteroid hormone with two forms, Vitamin $D_3$ and Vitamin $D_2$ (Gonnet, Lethuaut, & Boury, 2010). Particularly, Vitamin $D_3$ is responsible for various body functions, such as bone metabolism, intestinal transport, and blood pressure (Lind, Hänni, Lithell, Hvarfner, Sürensen, & Ljunghall, 1995; Nicolaysen, 1936). Vitamin E refers to a group of eight compounds which can be classified as four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ), and α-tocopherol is the principle Vitamin E in nature (Gonnet, Lethuaut, & Boury, 2010). Dietary Vitamin E exists in nuts, vegetables, seed oils and whole grains (Monsen, 2000). Various health benefits of Vitamin E have been studied. For example, Vitamin E can prevent membrane lipids from oxidation (Niki, Saito, Kawakami, & Kamiya, 1984), affects fertility positively (Rowlands & Singer, 1936), Vitamin E has anti-clotting activity (Dowd & Zheng, 1995). Vitamin K includes Vitamin $K_1$ and Vitamin $K_2$, it possesses antihemorrhagic properties (Dam, 1935).

### *1.1.2 Proteins*

Proteins are essential food ingredients as they are important sources of energy and essential and nonessential amino acids (Wu, 2009). Amino acids such as glutamine, arginine,

lysine, histidine, tyrosine and taurine play roles on many biochemical pathways and are precursors of bioactive metabolites (Damodaran, 2007). For proteinaceous foods, they also play a key role on the physicochemical and sensory properties. Furthermore, some dietary proteins are considered as potential ingredients for improving health due to their particular properties (H. Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998).

Dairy products are the principle source of bioactive proteins derived from foods (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Casein and whey proteins are two groups of proteins in milk. In bovine milk, about 80% of protein is casein that possesses various biological properties, for example, carriers of calcium, zinc, copper, iron and phosphate ions (H. J. Korhonen, 1995) and precursors of several bioactive peptides (Mulvihill & Fox, 1994). Whey proteins are composed mainly of $\beta$-lactoglobulin and $\alpha$-lactalbumin and secondary of immunoglobulins, glycomacropeptide, serum albumin, lactoferrin and numerous enzymes (Fox & Flynn, 1992). $\beta$-lactoglobulin is the carrier of rentiol, while $\alpha$-lactalbumin possesses immunomodulation and anticancer properties (H. J. Korhonen, 1995). Immunoglobulins and lactoferrin both have antimicrobial and antiviral properties, as well as modulating immune system (Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007).

In addition to dairy products, bioactive proteins are found in poultry and plant products (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Eggs are rich in proteins with various physicochemical and biological properties (Froning, 1994). Particularly, lysozyme, a protein from egg, is a food preservative that can decrease the pathogenic microorganisms on meat

surface and inhibit late fermentation of hard cheese (Li-Chan, Powrie, & Nakai, 1995). Plant bioactive proteins include zein from corn endosperm, glutelin and prolamin from rice, gluten from rice and soy protein (Ariyoshi, 1993; Phillips, 1997). For instance, soy proteins are able to reduce the risk of heart disease due to the decrease of blood lipid concentrations (H. Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998). However, the biological activities of these proteins are mostly enabled after producing specific peptides by enzymatic hydrolysis (Sarmadi & Ismail, 2010). For example, bioactive peptides produced from milk proteins (Table 1-1) possess antimicrobial, antithrombotic, and antihypertensive properties, etc. (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Peptides inhibiting angiotensin-coverting enzymes (ACE) were also detected after the hydrolysis of fish meat by pepsin, trypsin or chymotrypsin (Ariyoshi, 1993), $\alpha$-zein (a primary component of zein) by thermolysin (Miyoshi, Ishikawa, Kaneko, Fukui, Tanaka, & Maruyama, 1991). Other biological functions of peptides include mineral binding, antioxidative, hypochelesterolemic, immunomodulatory, and antithrombotic (Cross, Huq, Palamara, Perich, & Reynolds, 2005; Gauthier, Pouliot, & Saint-Sauveur, 2006; K. McCann, Shiell, Michalski, Lee, Wan, Roginski, et al., 2006; Zhong, Liu, Ma, & Shoemaker, 2007).

Enzymes are proteins in which biological property arises from an active site to catalyze chemical reactions (Pariza & Foster, 1983). Various enzymes have been applied in the food industry due to their functional activities. Transglutaminase is used as a texturing agent in the processing of noodles, yoghurt and sausages to enhance their viscoelastic properties (Kuraishi, Yamazaki, & Susa, 2001). Lactase is utilized to hydrolyze lactose in milk (Kirk, Borchert, &

Fuglsang, 2002). Neutrase is applied to speed up ripening of cheese (C. J. Kirby, Brooker, & Law, 1987). Besides, lactoperoxidase and lysozyme in dairy products have antimicrobial properties (de Wit, 1998).

### *1.1.3 Carbohydrates*

Carbohydrates with beneficial effects on health mainly refer to dietary fibers. According to the definition reported by the American Association of Cereal Chemists (AACC) (Camire, Cho, Craig, Devrie, Gordon, Jones, et al., 2001), "dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine". Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances (Table 1-2). Prebiotics, known as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host's health" (Collins & Gibson, 1999) are also dietary fibers.

The principle beneficial physiological effect of dietary fiber is laxation. Consumption of some dietary fibers would improve normal laxation due to the increase of stool weight (Cummings, 2001), which is attributed to the water holding properties of dietary fibers and the increase of microorganisms in stool produced by fermentation of dietary fibers (Kurasawa, Haack, & Marlett, 2000; Stephen & Cummings, 1980). Other beneficial influences of dietary fibers have also been found, such as reduction of colonic pH, and modification of intestinal microflora distribution (Camire, et al., 2001).

It has been confirmed that dietary fibers derived from certain food sources (for example, apple, barley, beans, fruits, vegetables and oatmeal), particular those fibers carried by low density lipoproteins (LDL), are able to decrease blood cholesterol levels (J. Marlett, Cho, & Dreher, 2001). This property is contributed to the viscosity of dietary fibers (J. A. Marlett, 1997). Fibers are the dominant compounds in gut lumen after digestion and absorption of foods in small intestine and increase the absorption of bile acid from ileum due to the viscosity. which reduces cholesterol in blood that is converted to bile acids by the liver in order to compensate the loss of bile acid in stool (Everson, Daggy, McKinley, & Story, 1992; J. A. Marlett, Hosig, Vollendorf, Shinnick, Haack, & Story, 1994).

With the increase of dietary fibers consumption, several beneficial influences are related to diabetics, such as improvement of glucose tolerance and peripheral tissue insulin sensitivity and reductions of insulin requirements, serum cholesterol and serum triglycerides (Anderson, Gustafson, Bryant, & Tietyen-Clark, 1987). Besides, fiber-rich diets play a positive role on weight control, because they possess a low energy density and high volume, which increase the time needed for digestion and absorption (Rolls, Bell, Castellanos, Chow, Pelkman, & Thorwart, 1999).

Other beneficial effects of dietary fibers have been also reported. Duodenal ulcers and cancer in the gastric cardia region can be prevented by consumption of dietary fibers (Aldoori, Giovannucci, Stampfer, Rimm, Wing, & Willett, 1997; Terry, Lagergren, Ye, Wolk, & Nyrén, 2001). Intestinal immune function is altered by the amount and type of fibers in diets according

to animal experiments (Field, McBurney, Massimino, Hayek, & Sunvold, 1999; Lim, Yamada, Nonaka, Kuramoto, Hung, & Sugano, 1997). It has been confirmed that modification of intestinal microflora populations will happen with the consumption of considerable quantities of non-digestible oligosaccharides (purified and homogenous fibers) (Mussatto & Mancilha, 2007). Fructooligosaccharides have the effect of improving calcium absorption (Coudray, Bellanger, Castiglia-Delavaud, Remesy, Vermorel, & Rayssignuier, 1997; van den Heuvel, Muys, van Dokkum, & Schaafsma, 1999).

### *1.1.4 Probiotics*

Probiotics are live microbial feed supplement beneficial to microorganism balance of the host (Anal & Singh, 2007). Various microorganisms from different genera are considered as probiotics (Table 1-3). However, probiotics mainly belong to *lactobacilli, enterococci* and *bifidobacteria* (Ouwehand, Salminen, & Isolauri, 2002). To date, the claimed health benefits of probiotics are improvement of lactose malabsorption (Savaiano, AbouElAnouar, Smith, & Levitt, 1984), reduction in incidence of antibiotic associated diarrhea (AAD) and plasma cholesterol concentration (McFarland, Surawicz, Greenberg, Elmer, Moyer, Melcher, et al., 1995; Surawicz, Elmer, Speelman, McFarland, Chinn, & Van Belle, 1989), suppression of cancer (Reddy, 1998), and enhancement of immune stimulation (Halpern, Vruwink, Van de Water, Keen, & Gershwin, 1991).

Addition of probiotic bacteria to foods has been verified to enhance the quantity of certain dietary nutrients. For example, lactic acid bacteria increased the content of folic acid,

niacin and riboflavin in fermented dairy products (Bronner & Pansu, 1999; Rajalakshmi & Vanaja, 1967; Tamime, 1981). Besides, enzymes and vitamins released by lactic acid bacteria are functional to alleviate intestinal malabsorption (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). For alleviation of lactose intolerance, lactic acid bacteria could improve the activity of lactase in intestine (Marteau, Flourie, Pochart, Chastang, Desjeux, & Rambaud, 1990).

Probiotics are widely used to prevent and treat diarrhea. To date, the claimed possible mechanisms for prevention of diarrhea by probiotic bacteria include their competition with pathogens for binding sites on epithelial cells and their production of bacteriocins to prohibit the growth of pathogen (Del Giudice & De Luca, 2004). For pediatric population, probiotic bacteria prevent viral diarrhea by enhancing the secretion of immunoglobulin A (Parvez, Malik, Ah Kang, & Kim, 2006).

Various studies adopted animal models to confirm the effect of probiotic bacteria on suppression of cancer (Reddy, 1998; Rowland, Rumney, Coutts, & Lievense, 1998). The mechanisms for the anti-tumor effect of probiotics are still not clear but have been speculated to involve stimulating immune system, removing fecal mutagens more effectively by changing colonic transit time, decreasing the pH of intestine to alter bacteria activity and bile solubility, and prohibiting the creation of carcinogens (McIntosh, 1996).

Probiotic supplements can influence the incidence of coronary heart diseases by altering plasma cholesterol concentration. A study concluded that serum LDL levels and total serum

cholesterol were significantly decreased by feeding 125 mL of probiotic milk daily (Schaafsma, Meuling, Van Dokkum, & Bouley, 1998). The possible mechanisms for this function are to interfere the absorption of cholesterol from gut, to absorb cholesterol directly, to affect the level of blood lipids by metabolites (Fooks, Fuller, & Gibson, 1999).

## 1.2 Challenges for application of bioactive ingredients into food products

In order to improve consumers' health by application of bioactive food ingredients, bioactive food ingredients have to keep their bioactivities during production, storage, transportation and consumption of foods. However, bioactive food ingredients are easy to become inactive and be degraded quickly under certain conditions. In this section, challenges for incorporation of bioactive food ingredients in food products are summarized.

### *1.2.1 Challenges for application of bioactive lipids*

One major concern for bioactive lipids is oxidation when exposed to light, heat, metals, enzymes and microflora (Shahidi & Zhong, 2010). Oxidative products of bioactive lipids can render some unpleasant flavors that affect the quality of food products (Frankel, 2014). It has been reported that oxidation of carotenoids would change their bioactive characteristics, included the reduced free radical-scavenging ability (Aust, Ale-Agha, Zhang, Wollersen, Sies, & Stahl, 2003). A study demonstrated that the oxidation product of β-sitosterol was harmful to Caco-2 cells (Ryan, Chopra, McCarthy, Maguire, & O'Brien, 2005). Antioxidant activity of fat soluble vitamins is decreased after oxidation (B. Chen, McClements, & Decker, 2013). Besides, low

water solubility of lipids limits the application in some water-based food products, for example, sauces, beverages, and dressing (B. Chen, McClements, & Decker, 2013).

Bioavailability of bioactive lipids refers to the quantity or portion of lipids absorbed by human after ingestion (Burton & Traber, 1990). Molecular weight, solubility, chemical reactivity and physical state all have effect on the bioavailability of lipids. For example, bioactive lipids in crystalline forms possess a lower bioavailability (B. Chen, McClements, & Decker, 2013). Due to the difference of isometric forms, conjugated linoleic acid possess different biological properties (Kennedy, Martinez, Schmidt, Mandrup, LaPoint, & McIntosh, 2010). It is necessary to improve the bioavailability of lipids with significant bioactivities but low digestibility, while it is equally important to reduce the bioavailability of lipids causing risks of cardiovascular diseases (David Julian McClements, Decker, & Park, 2008).

### *1.2.2 Challenges for application of bioactive proteins*

The application of bioactive food ingredients in food products has to maintain the original appearance, taste and texture of food products. However, some bioactive proteins and peptides possess a bitter or a nasty taste (Cho, Unklesbay, Hsieh, & Clarke, 2004; Friberg, Larsson, & Sjoblom, 2003), which is a limitation for their application in foods.

Biological characteristics of bioactive proteins can be altered by processes applied in food production. For example, glutamine and asparagine can be converted to glutamic acid and aspartic acid by acidic hydrolysis, while alkaline treatments can degrade cystine, serine and threonine (Anantharaman & Finot, 1993; Finot, 1997). Heat and ultra-high pressure processes

are additional factors impacting the bioactivity of proteins. High-pressure processing could deactivate enzymes (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998). During heating, the Maillard reaction occurs between lysine residues of proteins and reducing carbohydrates in the same food matrix, which can reduce the bioavailability of lysine and create unpleasant flavor (Finot, 1997). Furthermore, products from the Maillard reaction can have negative influence at the cellular level (Finot, 1997).

The bioactivity of some proteins will not be utilized until they reach the target sites of action. For example, in vivo physiological properties of bioactive peptides produced from milk have to exert after they reach the required site at the luminal side of intestinal tract (Meisel, 1997). Consequently, in order to fulfill the bioactivity, these proteins cannot be degraded before being transported to the target sites.

### *1.2.3 Challenges for application of bioactive carbohydrates*

When a certain quantity of dietary fibers is incorporated into food products in order to achieve the bioactivity of dietary fibers, one major concern is the negative effect on quality and sensory properties of products, including stability, appearance, texture, flavor and mouthfeel (David Julian McClements, Decker, Park, & Weiss, 2009). Another concern for the application of dietary fibers is the stability. For instance, in order to preserve the bioactivity, prebiotics in food products should be stable during food processing, such as high temperature and low pH conditions. However, some unfavorable reactions will happen under these harsh conditions. For example, the Maillard reaction will occur between prebiotics (reducing sugar) and amino acids at

high temperature, which will decrease the bioactivity of prebiotics (Huebner, Wehling, Parkhurst, & Hutkins, 2008). A study reported that the prebiotic activity score was reduced significantly when the fructo-oligosaccharides solution with 1% glycine at pH 4.0 was heated at 85 °C for 30 min, while no significant changes were observed after heating at pH 5.0-7.0 (Huebner, Wehling, Parkhurst, & Hutkins, 2008). Inulin was degraded significantly when dry heated at temperatures between 135 and 195°C up to 60 min (Böhm, Kaiser, Trebstein, & Henle, 2005; Böhm, Kleessen, & Henle, 2006).

### *1.2.4 Challenges for application of probiotics*

Incorporation of probiotics in fermented dairy products has been widely developed, but studies showed that it is difficult to keep the viability of probiotic bacteria during long term storage in commercial yoghurt products (Iwana, Masuda, Fujisawa, Suzuki, & Mitsuoka, 1993; Shah, Lankaputhra, Britz, & Kyle, 1995). Survivability of probiotics in yogurt is influenced by various parameters, such as oxygen content in yogurt, production of acid and hydrogen peroxide by yogurt bacteria, lack of nitrogen source derived from peptides or amino acids, and oxygen permeation ability of the package material (Dave & Shah, 1998; Hull, Roberts, & Mayes, 1984; Ishibashi & Shimamura, 1993; Iwana, Masuda, Fujisawa, Suzuki, & Mitsuoka, 1993). In addition to the poor viability of probiotics in traditional dairy products, probiotic supplement products in tablets or powder form also possess low survivability due to dehydration, oxygen and high temperature during drying (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010; Kailasapathy, 2002).

Preservation of viable probiotic cell counts is a fundamental requirement to fulfill their efficacy, because they need to survive during processing, transportation, and shelf life storage of food products, as well as harsh acidic conditions of the stomach and enzymes and bile salts in the small intestine before arriving the target sites in the gut (Kailasapathy, 2002). However, studies have confirmed that the functionalities of probiotics are expressed by some particular effector molecules or bioactive molecules located in the cell envelope of probiotics, and these effector molecules are glycoproteins (Kleerebezem & Vaughan, 2009; Konstantinov, Smidt, de Vos, Bruijns, Singh, Valence, et al., 2008; Van Baarlen, Troost, van Hemert, van der Meer, de Vos, de Groot, et al., 2009). It has been concluded that preservation of these effector proteins in probiotics during processing, transport, storage and gastrointestinal tract is more important than preservation of their viability (Konstantinov, et al., 2008). Consequently, protection of effector molecules in probiotic bacteria is considered as a vital challenge for application of probiotics (Ledeboer, Nauta, Sikkema, Laulund, Niederberger, & Sijbesma, 2006).

### 1.3 An overview of colloidal delivery systems

Colloidal delivery systems are widely utilized in the food industry to overcome the side effects produced by incorporation of bioactive food ingredients and to preserve bioactivity of bioactive food ingredients during processing, storage and passing through gastrointestinal tract (Velikov & Pelan, 2008). In this section, traditional colloidal delivery systems for food ingredients are briefly reviewed.

### *1.3.1 Liposomes*

Originally, liposomes were fabricated for drug delivery in 1970s (Musthaba, Baboota, Ahmed, Ahuja, & Ali, 2009). Liposomes are self-assembled vesicular structures with one or more bilayers of phospholipids surrounding aqueous phase (Patel & Velikov, 2011). According to the amount of bilayer membranes, liposomes are categorized as small unilamellar vesicles (<30 nm) (SUV), large unilamellar vesicles (20-100 nm) (LUV) and multilamellar vesicles (MLV) (New, 1990). Due to the existence of hydrophobic palisade and hydrophilic compartment, liposomes are not only able to delivery water-soluble but also water-insoluble compounds (Gregory, 2006). Liposomes can prevent water-soluble bioactive ingredients from degradation and increase the solubility of lipophilic bioactive ingredients in aqueous solution (Gregory, 2006).

Apart from the pharmaceutical industry, more and more investigations have applied liposomes for potential food applications. For example, cohesiveness and elasticity of Cheddar cheeses were increased after application of liposome encapsulating lipase (Kheadr, Vuillemard, & El‐Deeb, 2002). Liposome-entrapment could significantly increase the recovery of vitamin D in cheeses (Banville, Vuillemard, & Lacroix, 2000). The activity of α–amylase entrapped in liposomes prepared with lecithin/cholesterol was preserved effectively in acidic (pH 2.8) and pepsin conditions (Hsieh, Chen, Wang, Chang, & Chang, 2002). Phosphatidylcholine liposomes improved the antioxidant activity of ascorbic acid, as demonstrated for more than 50% residual activity after 50-day storage in a refrigerator that contrasted with no activity of free ascorbic acid

after 19 days (C. Kirby, Whittle, Rigby, Coxon, & Law, 1991). Liposome-encapsulation retarded the degradation of retinol under either light or heat condition (S.-C. Lee, Yuk, Lee, Lee, Hwang, & Ludescher, 2002).

### *1.3.2 Emulsions*

Emulsions are dispersions fabricated from two immiscible liquids in the presence of surfactants (Nielloud, 2000). Typically, the droplet size of emulsions ranges from 0.1 to 100 μm (David Julian McClements & Li, 2010). Based on the droplet dimension, emulsions are divided into two groups, nanoemulsions (50-400 nm) and emulsions (Patel & Velikov, 2011; Velikov & Pelan, 2008). Nanoemulsions and emulsions are not thermodynamically stable (Nielloud, 2000). According to the relative location of aqueous and oil phases within the dispersion, different emulsion structures can be fabricated (D. McClements & Decker, 2000). Oil-in-water (O/W) emulsions have oil droplets dispersed in the water phase, while water-in-oil (W/O) emulsions are composed of water droplets dispersed in the oil phase. When solid lipids are applied to fabricate emulsions, solid lipid particles (SLP) are produced (D Julian McClements, Decker, & Weiss, 2007). Additionally, various multiple emulsions have been developed, such as water-in-oil-in-water (W/O/W) emulsions, oil-in-water-in-oil (O/W/O) emulsions (Aoki, Decker, & McClements, 2005; Güzey & McClements, 2006).

Emulsions are utilized as common colloidal delivery system since they can be simply created by some food grade ingredients (Acosta, 2009; David Julian McClements, Decker, Park, & Weiss, 2009). Particularly, emulsion-based delivery systems have been widely applied to

encapsulate, control and release bioactive ingredients with poor water solubility (Chakraborty, Shukla, Mishra, & Singh, 2009; David Julian McClements & Li, 2010). For example, O/W emulsions of ω-3 fatty acids can be used to fortify food products and protect lipids from oxidation (Chee, Gallaher, Djordjevic, Faraji, McClements, Decker, et al., 2005; S. Lee, Decker, Faustman, & Mancini, 2005). Tuna oil as a good source of ω-3 fatty acids was encapsulated by multilayered emulsion prepared with lecithin and chitosan (Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005). For this system, a primary emulsion was fabricated by mixing tuna oil and lecithin aqueous solution by homogenization at pH 3.0, and a secondary emulsion was prepared by mixing the primary emulsion with chitosan solution to adsorb cationic chitosan on primary oil droplets with anionic lecithin. The secondary emulsion prevented tuna oil from oxidation better than the primary emulsion did (Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005). W/O/W emulsions have been applied to encapsulate hydrophilic bioactive ingredients. For instance, vitamin B2 was encapsulated in W/O/W emulsions by a two-stage homogenization method, initially forming a W/O emulsion with kerosene oil phase and Span 80, followed by emulsifying the W/O emulsion into a Tween 20 aqueous solution (Owusu, Zhu, & Dickinson, 1992). Immunoglobulin G was also encapsulated in W/O/W emulsions by first homogenizing the immunoglobulin G aqueous solution in an oil phase with soybean oil and Span 80 to fabricate W/O emulsion that was then homogenized in an aqueous solution with soy protein, sucrose stearate or Tween 80 to create W/O/W emulsions (C.-C. Chen, Tu, & Chang, 1999).

### *1.3.3 Micelles*

Micelles are self-assembled structures of amphiphilic molecules dissolved at a concentration above the critical micelle concentration (CMC) (Sagalowicz & Leser, 2010). Amphiphilic molecules are composed of a hydrophilic head (polar moiety) and a hydrophobic tail (nonpolar moiety) (Rangel-Yagui, Pessoa Jr, & Tavares, 2005). The hydrophobic tails are located in the core of a micelle so as to avoid the contact with water, and the hydrophilic heads tend to stay on the surface to contact with water (Chevalier & Zemb, 1990). Micelles are thermodynamically stable and have a size typically smaller than 10 nm and are utilized as delivery systems extensively (Boyd, 2008). For instance, curcumin encapsulated in camel beta-casein micelles had a higher antioxidant activity than free curcumin, as well as the cytotoxicity to cancer cell (Boyd, 2008). Casein micelles also have been applied to encapsulate vitamin D2 to prevent the UV-light degradation (Semo, Kesselman, Danino, & Livney, 2007). Phospholipid micelles were studied to enhance the cellar uptake of carotenoids (Sugawara, Kushiro, Zhang, Nara, Ono, & Nagao, 2001).

### *1.3.4 Nanoparticles*

Nanoparticles are nanometeric systems that include solid lipid nanoparticles (SLNs) and polymeric nanoparticles (Patel & Velikov, 2011). SLNs are similar to conventional O/W emulsions, but with a solid lipid core (Jochen Weiss, Decker, McClements, Kristbergsson, Helgason, & Awad, 2008). SLNs are composed of solid lipids, single or multiple surfactants, and water. A mixture of surfactants is used to fabricate SLNs due to the better emulsifying property

(Jenning, Mäder, & Gohla, 2000). SLNs have been studied widely for both food and pharmaceutical applications, since solid lipids had a better ability to control the release and the stability of bioactive compounds than liquid lipids due to the manipulation of mobility of bioactive compounds (Mehnert & Mäder, 2001). Hot homogenization method has been used to fabricated SLNs to encapsulation of lipophilic vitamins, and features such as the protection of vitamins under certain conditions and delivery to target sites have been reported (Işcan, Wissing, & Müller, 2005; Jenning, Gysler, Schäfer-Korting, & Gohla, 2000; Pople & Singh, 2006; Saupe, Wissing, Lenk, Schmidt, & Müller, 2004). SLNs are also considered as potential carriers for bioactive peptides and proteins to protect the stability of these compounds and prevent their degradation by enzymes (Almeida & Souto, 2007).

Polymeric nanoparticles are colloidal systems fabricated by synthetic or natural polymers (Patel & Velikov, 2011). They have been recognized as favorable delivery systems for various bioactive ingredients and medicinal drugs due to their particular characteristics, such as subcellular size, ability to control or sustain release and biodegradation or biocompatibility (Kumari, Yadav, & Yadav, 2010). Polymers commonly used for polymeric nanoparticles include poly acrylic acid family polymers, poly (lactic acid), poly(D,L-glycolide), polysaccharides (particularly chitosan), proteins or polypeptides (such as gelatin) (Kumari, Yadav, & Yadav, 2010; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001). For instance, chitosan was developed to be a biopolymeric delivery system to decrease the release of catechin in the gastrointestinal tract but release the rest of catechin in the large intestine (Zhang & Kosaraju,

2007). Quaternized chitosan and alginate nanoparticles were fabricated for the oral delivery of bovine serum albumin (Li, Shi, Du, & Tang, 2007). Plasmid DNA was encapsulated in chitosan/ poly acrylic acid nanoparticles, due to the opposite charges of these two polymers, as potential gene delivery systems (Q. Chen, Hu, Chen, Jiang, & Yang, 2005).

### 1.4 Hypothesis and overview of dissertation research

The overall hypothesis of this dissertation is that solid/oil/water (S/O/W) emulsions can be used to encapsulate water-soluble/dispersible ingredients to retain their release during processing and storage and control their release during simulated digestions. Bioactive food ingredients studied in this research are L-glutamine, probiotic *Lactobacillus salivarius* NRRL B-30514, and lactase. For food applications, delivery systems should be fabricated using food grade ingredients (Lutz, Aserin, Wicker, & Garti, 2009; Samson, Dube, Aucoin, Wierzbicki, Maroun, Letourneau, et al., 2013; J. Weiss, Takhistov, & McClements, 2006). Phospholipids and surface active proteins and polysaccharides are common choices of emulsifiers (Guzey & McClements, 2006). Whey protein isolate (WPI) and sodium caseinate (NaCas) are commercially available food ingredients extensively used to prepare emulsions. For example, NaCas and WPI have been used as the outer emulsifiers in W/O/W emulsions (Benichou, Aserin, & Garti, 2007b; Bonnet, Cansell, Berkaoui, Ropers, Anton, & Leal-Calderon, 2009), and WPI has been studied as the emulsifier for both interfaces in O/W/O emulsions (Benichou, Aserin, & Garti, 2007a). The stability of emulsions prepared by WPI and NaCas is affected by thermal, ionic and acidic stresses (Chanamai & McClements, 2002; Dickinson & Davies, 1999; Kim, Decker, &

McClements, 2002), which may be improved by preparing multiple-layered emulsions, e.g., by depositing pectin on protein-coated droplets at acidity below protein isoelectric point (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Besides, sugar beet pectin (SBP) has poor gelling ability but has good emulsifying properties due to the protein segment glycated to polysaccharides and the acetyl group (Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005). Therefore, SBP is a potential polymeric surfactant to fabricate S/O/W emulsion.

It was hypothesized that S/O/W emulsion, as a delivery system, could prevent the degradation of water soluble compounds during processing, storage and harsh conditions. Consequently, in chapter 2, multiple-layered S/O/W delivery systems were fabricated to minimize the release of glutamine (a water soluble conditionally essential amino acid) during storage and gastric digestion and enable the gradual and complete release during intestinal digestion. Structurally, spray-dried glutamine solid particles were encapsulated with S/O/W emulsions with sequential protein (WPI or NaCas) and citrus pectin interfacial layers. Release of glutamine during storage at 4 ºC and in simulated gastric fluid was determined.

In chapter 3, it was hypothesized that double-layered S/O/W emulsion could enhance the survival of spray-dried probiotic *L. salivarius* NRRL B-30514 according to the conclusions obtained from chapter 2. The spray-dried cells were encapsulated in the double-layered emulsions, and viability of encapsulated bacteria was determined during storage at 4 ºC, after heating, and in vitro digestion.

Normally, pectin is widely used as a food additive for gelling and stabilizing functions. SBP has poor gelling ability but has good emulsifying properties due to the protein segment glycated to polysaccharides and the acetyl group. In chapter 4, it was assumed that SBP, as a polymeric surfactant, was able to fabricate S/O/W emulsions to improve the viability of spray-dried *L. salivarius* cells. The viabilities of encapsulated *L. salivarius* during storage, after thermal treatment, and in vitro digestion were tested.

It was hypothesized that survival of *L. salivarius* after spray drying and storage can be affected by various factors, such as drying media, heat adaptation and outlet temperature. In chapter 5, *L. salivarius* were spray-dried with or without prior heat adaptation, with different drying media and outlet temperatures, the viabilities of spray-dried bacteria after spray drying and subsequent storage in a desiccator at 21 ºC were determined.

In chapter 6, drying media and water activity of spray-dried *L. salivarius* were assumed to be related to the viability of *L. salivarius* after encapsulating by S/O/W emulsion under certain conditions, optimized drying media and water activity of dried cell could increase the viable cell counts of encapsulated spray-dried cells as many as possible. Spray-dried *L. salivarius* with different drying media and water activities were encapsulated by S/O/W emulsion prepared with SBP, and viability of encapsulated *L. salivarius* was tested during storage at 4 ºC or 22 ºC and pasteurization.

S/O/W emulsion could be a potential delivery system of lactase for producing lactose free milk without changing its original flavor. In chapter 7, it was hypothesized that S/O/W emulsion

was able to preserve the activity of lactase during thermal processing and storage. With encapsulated lactase, lactose hydrolysis in milk was diminished during refrigerator storage, but was increased during simulated gastric and intestinal digestions. Lactase were encapsulated by S/O/W emulsions prepared with WPI or NaCas. The activity of encapsulated lactase during processing and storage was tested. The release of lactase in simulated gastrointestinal condition and the hydrolysis of lactose in milk with encapsulated lactase during in vitro digestion were also evaluated.

S/O/W emulsions studied in this dissertation can be used to control the release and/or activity of water-soluble ingredients and water-dispersible colloidal particles such as probiotics during processing, storage, and post-ingestion. For probiotics, these emulsion systems can be used to enhance the viability of cells during storage, after pasteurization, after drying, and after simulated gastrointestinal digestion. For lactase, S/O/W emulsions can be used to enhance the enzymatic activity after heating and drying and reduce the hydrolysis of lactose in milk during storage, while enhancing lactose hydrolysis in simulated gastrointestinal conditions. Consequently, these S/O/W emulsions are potential delivery systems to incorporate bioactive food ingredients in the food industry.

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## Appendix

Table 1-1. Bioactive peptides derived from milk proteins (H. Korhonen, et al., 1998).

| Bioactive peptides | Protein precursor | Bioactivity |
|---|---|---|
| Casomorphins | $\alpha$- and $\beta$-Casein | Opioid agonists |
| $\alpha$-Lactorphin | $\alpha$-Lactalbumin | Opioid agonist |
| $\beta$-Lactorphin | $\beta$-Lactoglobulin | Opioid agonist |
| Lactoferroxins | Lactoferrin | Opioid antagonists |
| Casoxins | $\kappa$-Casein | Opioid antagonists |
| Casokinins | $\alpha$- and $\beta$-Casein | Antihypertensive |
| Casoplatelins | $\kappa$-Casein, Transferrin | Antithrombotic |
| Immunopeptides | $\alpha$- and $\beta$-Casein | Immunostimulants |
| Phosphopeptides | $\alpha$- and $\beta$-Casein | Mineral carriers |
| Lactoferricin | Lactoferrin | Antimicrobial |

Table 1-2. Constituents of dietary fiber (Camire, et al., 2001).

| |
|---|
| ***Polysaccharides and resistant oligosaccharides*** |
| Cellulose |
| Hemicellulose |
| Pectin |
| Gums |
| Mucilages |
| Resistant starch |
| Fructans, oligofructans |
| Galactooligosaccharides |
| ***Analogous carbohydrates*** |
| Indigestible dextrins (obtained by hydrolysis of starch) |
| Resistant maltodextrins and resistant potato dextrins |
| Synthetic carbohydrates (polydextrose, methyl cellulose and hydroxypropylmethyl cellulose) |
| ***Lignin*** |
| ***Associated plant substances*** |
| Waxes |
| Phytate |
| Cutin |
| Saponins |
| Suberin |
| Tannins |

Table 1-3. Microbes used as probiotics and their reported health benefits in human clinical trials[a]

| Genus | Species | Example strains | Health benefits |
|---|---|---|---|
| *Lactobacillus* | *acidophilus* | La5 | Reduced antibiotic associated diarrhoea |
| | *casei* | Shirota | Shortening of rotavirus diarrhoea Reduced recurrence of superficial bladder cancer |
| | | | Immune modulation |
| | *johnsonii* | La1 | Improved oral vaccination |
| | | | Reduced colonization by *Helicobacter pylori* |
| | *plantarum* | 299v | Relief of irritable bowel syndrome Reduction of LDL-cholesterol |
| | *reuteri* | SD2112 | Shortening of rotavirus diarrhoea |
| | *rhamnosus* | GG | Shortening of rotavirus diarrhoea Immune modulation |
| | | | Relief of inflammatory bowel disease |
| | | | Treatment and prevention of allergy |
| | *salivarius* | UCC118 | Reduced symptoms of inflammatory bowel disease |
| *Bifidobacterium* | breve | | Reduced symptoms of irritable bowel disease |
| | *lactis* | Bb12 | Treatment of allergy Shortening of rotavirus diarrhoea Reduced incidence of travellers diarrhoea Improved oral vaccination |
| *Escherichia* | *coli* | Nissle 1917 | Fewer relapses of inflammatory bowel disease |
| *Saccharomyces* | *cerevisiae* | boulardii | Fewer relapses of inflammatory bowel disease |

[a]Table is adapted from Ouwehand (2002) with modification.

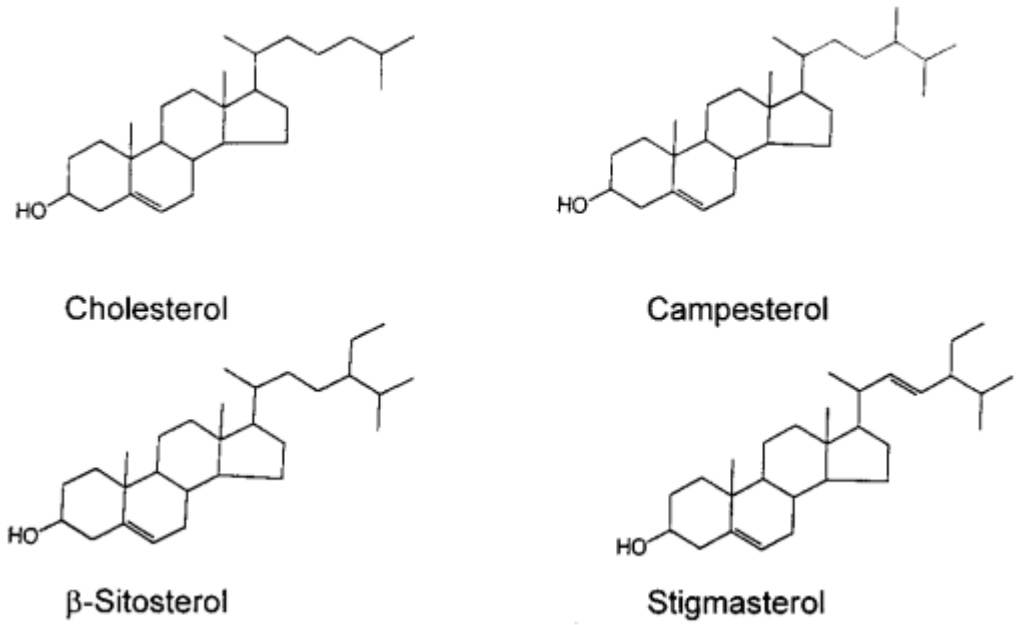


Figure 1-1. Chemical structures of sterols(Awad & Fink, 2000).

# Chapter 2 . Multiple-layered coatings on L-glutamine solid microparticles for the retention during storage and enteric delivery during *in vitro* digestions

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## 2.1. Abstract

The objective of this work was to study delivery systems of water-soluble bioactive compounds using solid/oil/water emulsions to minimize the release during storage and gastric digestion. Spray-dried glutamine solid particles were suspended in milk fat that was emulsified by whey protein isolate or sodium caseinate at neutral pH as primary emulsions, followed by deposition of pectin at pH 3.0 to obtain secondary emulsions. Zeta-potential results verified the preparation of secondary emulsions. Secondary emulsions showed a higher efficiency of encapsulating glutamine and retaining glutamine during storage. For emulsions reconstituted from spray dried secondary emulsions, <20% release of glutamine was observed at pH 2.0-7.0 after 2-week refrigerated storage. The secondary emulsions retained glutamine during the 2-h simulated gastric digestion better than primary emulsions and showed gradual release to >90% after the subsequent 4-h simulated intestinal digestion. Therefore, the solid/oil/water secondary emulsions can be used as delivery systems of water-soluble bioactive compounds.

**Keywords**: L-glutamine, multiple-layered coatings, protein, pectin, storage stability, enteric delivery

## 2.2. Introduction

Bioactive food components, although present in small quantities, provide beneficial effects on health by influencing physiological or cellular activities (P. M. Kris-Etherton, et al., 2004). Consequently, it is important to supplement bioactive compounds in food products. Bioactive compounds include a diverse range of substances, including fatty acids, carotenoids, polyphenols, phytosterols, probiotics, and amino acids (Champagne & Fustier, 2007; S.-K. Kim & Mendis, 2006; Penny M. Kris-Etherton, et al., 2002; Lukaski, 2004; Rodriguez-Huezo, Pedroza-Islas, Prado-Barragan, Beristain, & Vernon-Carter, 2004). However, physical, chemical, and biochemical conditions during food processing and storage and physiological conditions after ingestion can cause structural changes of bioactive compounds and therefore the loss of functionality (Ubbink & Kruger, 2006). Delivery systems are studied to improve the stability of bioactive compounds before consumption and the availability at absorption sites after ingestion to eventually enhance the *in vivo* bioavailability and bioactivity (D. J. McClements, Decker, Park, & Weiss, 2009).

Various emulsion-based delivery systems have been fabricated to control the release and improve the stability of bioactive components. This has led to studies using the layer-by-layer deposition technique that fabricates the interface of oil-in-water (O/W) emulsions with multiple layers of oppositely-charged compounds, which can protect the encapsulated compounds and control the digestion (Benjamin, Silcock, Leus, & Everett, 2012; Chen & McCarthy, 1997; Guzey & McClements, 2006; D. J. McClements & Li, 2010). In this approach, the primary emulsion is usually prepared by homogenizing lipids in the aqueous phase with an ionic

emulsifier. The secondary emulsion is created by adding to the primary emulsion a biopolymer with opposite charges to the ionic emulsifier, and the adsorption of biopolymers onto the surface of primary emulsion droplets forms double-layered interfaces (Aoki, Decker, & McClements, 2005). The process can be repeated to prepare multiple-layered emulsions that can be engineered to have strong repulsive electrostatic and steric forces to prevent droplet aggregation and protect labile compounds against environmental stresses such as pH, temperature and enzymes during processing, storage, and digestion (Benjamin, et al., 2012). Another advancement is the multiple water/oil/water (W/O/W) emulsions that allow the encapsulation of both hydrophilic (in inner water droplets) and hydrophobic (in the oil phase) compounds in one droplet. The stability of inner water droplets is a concern in W/O/W emulsions (Vasiljevic, Parojcic, Primorac, & Vuleta, 2006).

For food applications, delivery systems should be fabricated using food grade ingredients (Lutz, Aserin, Wicker, & Garti, 2009; Samson, et al., 2013; Weiss, Takhistov, & McClements, 2006). Phospholipids and surface active proteins and polysaccharides are common choices of emulsifiers (Guzey, et al., 2006). Whey protein isolate (WPI) and sodium caseinate (NaCas) are commercially available food ingredients extensively used to prepare emulsions. For example, NaCas and WPI have been used as the outer emulsifiers in W/O/W emulsions (A. Benichou, Aserin, & Garti, 2007b; Bonnet, et al., 2009), and WPI has been studied as the emulsifier for both interfaces in oil/water/oil emulsions (A. Benichou, Aserin, & Garti, 2007a). WPI is composed of mostly globular β-lactoglobulin with an isoelectric point (pI) of 5.2-5.3, α-lactalbumin (pI = 4.2), and bovine serum albumin (pI = 4.9) (Bramaud, Aimar, & Daufin, 1997;

Euston, Singh, Munro, & Dalgleish, 1996; Kaibara, Okazaki, Bohidar, & Dubin, 2000; Townend, Weinberger, & Timasheff, 1960). NaCas is a mixture of four types of caseins ($\alpha_{s1}$, $\alpha_{s2}$, β, and κ), of which β- and $\alpha_{s1}$-caseins make up more than 75% of the total protein and are the major contributors of emulsifying properties of NaCas (Robson & Dalgleish, 1987). Compared with WPI, NaCas is disordered and is more surface active and thus a better emulsifier (Eric Dickinson, Rolfe, & Dalgleish, 1988; Euston, Singh, Munro, & Dalgleish, 1995). The stability of emulsions prepared by WPI and NaCas is affected by thermal, ionic and acidic stresses (Chanamai & McClements, 2002; Eric Dickinson & Davies, 1999; H. J. Kim, Decker, & McClements, 2002), which may be improved by preparing multiple-layered emulsions, e.g., by depositing pectin on protein-coated droplets at acidity below protein pI (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998).

Compared to numerous systems studied for lipophilic compounds, there is little information about delivery systems that incorporate small water-soluble compounds in aqueous systems. Although W/O/W emulsions are principally feasible to deliver water-soluble compounds, the need to dissolve compounds in the inner water phase lowers the loading level, in addition to the instability concerns discussed above. To address these challenges, the objective of this work was to fabricate multiple-layered solid/oil/water (S/O/W) delivery systems that minimize the release of water-soluble compounds during storage and gastric digestion and enable the gradual and complete release during intestinal digestion. Structurally, spray-dried glutamine solid particles were encapsulated within anhydrous milk fat (MF) that was emulsified by WPI or NaCas, followed by depositing pectin at pH 3.0. In our previous study, we demonstrated that

glutamine as received was rod-shaped crystals with a length larger than 100 μm and can be converted to spheres with an average diameter of 3.8 μm after dissolving in water and spray drying (Zhong & Shah, 2013). Spherical particles could facilitate the encapsulation in the lipid phase during homogenization. Glutamine, with a molecular weight of 146.1 Da and water solubility of 35 g/L at 20 ℃ (Fürst, 2001), was chosen as a model compound because it is a conditionally essential amino acid with profound functions in the physiology of humans and animals (Lacey & Wilmore, 1990). In addition to being a carrier of nitrogen, carbon and energy between organs (Curthoys & Watford, 1995), glutamine has anti-inflammation functions and can stimulate immune cells with anti-cancer significance (Medina, 2001; Newsholme, 2001). However, glutamine is heat-labile in aqueous solutions and is easily converted to glutamic acid during heating at acidic conditions (Lacey, et al., 1990). Additionally, it is glutamine not glutamic acid that is needed to fulfill its roles as a conditionally essential amino acid at stressed conditions (Wu, 1998). The studied delivery system may prevent the degradation of water-soluble compounds during storage in acidic foods and in gastric juice after ingestion and enable their gradual release during intestinal digestion with neutral acidity.

## 2.3. Materials and methods

### *2.3.1. Materials*

WPI (93.4% protein, dry basis) was provided by Hilmar Ingredients (Hilmar, CA, USA). NaCas (93% protein, dry basis) was purchased from American Casein Co. (Burlington, NJ, USA). Pectin from citrus peel (galacturonic acid ≥74.0%, dry basis) and lipase from porcine

pancreas were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Anhydrous MF was provided by Land O'Lakes, Inc. (Arden Hills, MN, USA). Other chemicals were from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific (Pittsburgh, PA, USA).

### *2.3.2. Preparation of emulsions*

To prepare multiple-layered S/O/W emulsions, spray-dried glutamine powder was prepared as previously described (Zhong, et al., 2013) and was suspended at 6.25% w/v in melted MF at 40 ℃ and stirred until a uniform suspension was obtained. Protein (WPI or NaCas) and pectin solutions were prepared separately at 1.0% w/v and adjusted to pH 7.0 using 1 M NaOH, with sodium azide added at 0.02% w/v as an antimicrobial agent. Primary emulsions were prepared by homogenizing the glutamine-MF suspension at O:W phase volume ratios of 1:5, 1:7 and 1:9 in the protein solution at 12,000 rpm for 2 min using a high speed homogenizer (model Cyclone I.Q.$^2$, The VirTis Co., Inc., Gardiner, NY, USA). The secondary emulsions were prepared by mixing the primary emulsion with the pectin solution at a volume ratio of 1:1, followed by adjusting pH to 3.0 using 1 M HCl. The prepared emulsions were stored at 4 ℃ prior to analyses.

### *2.3.3. Encapsulation efficiency*

Encapsulation efficiency was determined by indirectly measuring the glutamine content in the aqueous phase (free glutamine). The emulsion samples were centrifuged at 13,000 rpm for 2 min to precipitate droplets with glutamine solid particles using a MiniSpin Plus centrifuge (Eppendorf, Inc. Hauppauge, NY, USA), and the serum was determined for glutamine

concentration using a colorimetric method detailed previously (Zhong, et al., 2013). The encapsulation efficiency was calculated according to the following equation.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{total glutamine (g)} - \text{free glutamine (g)}}{\text{total glutamine (g)}} \times 100\% \qquad (2\text{-}1)$$

### *2.3.4. Particle size analysis*

The dimension of droplets was measured using a laser diffraction particle size analyzer (model LS 13 320, Beckman, Brea, CA, USA) for primary and secondary emulsions after overnight storage at 4 ℃. The arithmetic mean diameter ($d_{1,0}$) of droplets was reported.

### *2.3.5. Retention of glutamine during storage*

Primary and secondary emulsions were stored at 4 ℃. Emulsion samples (1.5 mL) were sampled at a preset time point (1, 7, and 14 days) and centrifuged to obtain the supernatant to indirectly quantify percentages of glutamine that remained encapsulated, as in the determination of encapsulation efficiency.

### *2.3.6. Preparation of spray-dried capsules*

The emulsions were spray-dried using a Mini Spray Dryer (model B290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) with a pump rate set at 10%. The inlet air temperature was 165 ℃, and the outlet temperature was 90 ℃.

### *2.3.7. Release of glutamine in suspensions reconstituted with spray-dried capsules*

Reconstituted emulsions were prepared by suspending 0.05 g spray-dried powder in 10 mL distilled water and were adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 using 1 M HCl or 1 M

NaOH. Reconstituted emulsions were kept at 4 ℃ in a refrigerator before the following experiments.

#### *2.3.7.1. Optical microscopy*

The microstructure of reconstituted emulsions was observed using a BX60 microscope (Olympus Latin America, Inc., Miami, FL, USA)

#### *2.3.7.2. Zeta potential*

The zeta potential of emulsions was measured using a Delsa Nano instrument (Beckman Coulter Inc., Fullerton, CA, USA). The reconstituted emulsion (1 mL) was transferred to a 1.5 mL centrifuge tube, and centrifuged at 13,000*g* for 2 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany) to remove free molecules. The precipitate was re-suspended in distilled water adjusted to pH 2.0-7.0 using 1 M HCl or 1 M NaOH and was diluted 50 times with a phosphate buffered saline at the same pH before zeta potential measurement.

#### *2.3.7.3. Release of glutamine during storage*

Emulsion samples were stored at 4 ℃ up to 14 days. At a preset time point (0, 7 or 14 days), 1.5 mL of a suspension was sampled and centrifuged to determine free glutamine as above.

#### *2.3.7.4. Determination of free pectin in reconstituted emulsions*

Reconstituted emulsions were withdrawn and centrifuged at 14,000*g* for 2 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany), and the supernatant was collected to determine

free pectin using a colorimetric method (Ye, Flanagan, & Singh, 2006). Standard pectin solutions were used to establish a calibration curve.

### *2.3.8. In vitro release kinetics during simulated digestions*

Both fresh and reconstituted emulsion samples were evaluated for release kinetics during the simulated *in vitro* digestions. Simulated gastrointestinal fluids were prepared using the literature formulations (Annan, Borza, & Hansen, 2008; Sarkar, Goh, Singh, & Singh, 2009). The simulated gastric fluid contained 3 g/L pepsin in physiological saline (with 8.5 g/L NaCl), and the pH was adjusted to 2.0 with 1 M HCl. Two mL of freshly prepared emulsions or 0.1 g spray-dried powder was mixed with 15 mL of the simulated gastric fluid. After incubation in a shaking water bath (New Brunswick Scientific Co., Edison, NJ, USA) at 37 ℃ for 1 and 2 h, 1 mL of the mixture was withdrawn and centrifuged at 14,000*g* for 2 min, and 0.2 mL of the supernatant was collected for the colorimetric assay of glutamine concentration. In order to simulate the subsequent intestinal digestion, the bile salt, lipase, trypsin and pancreatin were added at 10.0, 0.0025, 0.0025, and 1.0 g/L, respectively, in the emulsions after the 2-h simulated gastric digestion, and the mixture pH was adjusted to 6.8 using 1 M NaOH. The mixture was then incubated in the shaking water bath at 37 ℃ for 1, 2, 3, and 4 h before sampling for analysis of the released glutamine content as described previously.

Preliminary experiments showed that free glutamine was not hydrolyzed during *in vitro* gastric and intestinal digestions. To confirm no glutamine was produced from hydrolysis of proteins, control emulsions were prepared as above without glutamine, and no glutamine was

detected after digestions and assayed using the above method. Therefore, the above protocol only measured glutamine released from capsules.

### *2.3.9. Statistical analysis*

Mean and standard deviation were calculated from triplicates. One-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL). The least-significant-difference (LSD) mean separation method was used to analyze statistical differences of the mean at a *P* level of 0.05.

## 2.4. Results and discussion

### *2.4.1. Droplet dimension of fresh emulsions*

The arithmetic mean diameter ($d_{1,0}$) of emulsions after overnight storage is shown in Table 2-1. Overall, bigger droplets were observed at a higher volume fraction of oil phase, which likely is due to the reduced availability of protein in the continuous aqueous phase increasing the possibility of coalescence during emulsification (Pandolfe, 1995). Primary emulsions overall had bigger droplets than secondary emulsions. It was previously reported that pectin can inhibit the aggregation of emulsions prepared by NaCas (Douglas G Dalgleish & Hollocou, 1997). Emulsions prepared with NaCas had bigger droplets than those of WPI. This can be explained by studies showing emulsions prepared with whey proteins have better stability against aggregation and coalescence than those of caseins, because whey proteins denature after adsorption on the interface and form thicker and more elastic interfacial layers (Færgemand, Murray, & Dickinson, 1997) (Eric Dickinson, 2001).

### *2.3.2. Encapsulation efficiency and storage stability of fresh emulsions*

Encapsulation efficiency is summarized in Table 2-2. For WPI-based emulsions, an encapsulation efficiency of >92% was observed for all treatments, and no significant difference was observed between primary and secondary emulsions ($P > 0.05$). Conversely, a lower encapsulation efficiency was observed when a smaller volume of NaCas solution was used, and secondary emulsions had a significantly higher encapsulation efficiency than primary emulsions when the O:W phase volume ratio was 1:5 ($P < 0.05$). Because secondary emulsions were prepared based on primary emulsions, the encapsulation efficiency of primary emulsions is not expected to be lower than those of secondary emulsions if the interface and MF layer structures are stable. Therefore, it is likely that glutamine in NaCas-emulsified primary emulsions was released to some extent before quantification of free glutamine, but the deposition of pectin on NaCas-coated droplets stabilized the interface resulting in the higher encapsulation efficiency quantified in secondary emulsions than that in primary emulsions. This agrees with an earlier study where volatile compounds were encapsulated in primary and secondary emulsions (Benjamin, et al., 2012).

Figure 2-1 demonstrates percentages of glutamine that remained encapsulated during storage at 4 ℃. Overall, primary emulsions were poorer than secondary emulsions in retaining glutamine, and glutamine was kept in WPI-based emulsions better than those prepared with NaCas. After 14-day storage, emulsions with O:W phase volume ratio of 1:9 had the highest retention of glutamine and was therefore chosen for further studies. The emulsions prepared at

this ratio using WPI as an emulsifier showed the best performance, with 85.3% and 90.3% glutamine retained after 14-day in primary and secondary emulsions, respectively.

Although present as a solid at room and refrigerated temperatures, MF is a complex mixture composed of more than 400 saturated and unsaturated fatty acids, such as oleic, palmitic, myristic and stearic acids (Ulberth & Lees, 2003). Therefore, some fluidity of the MF layer outside glutamine solid particles is expected. The better stability of double-layered than single-layered interfaces is well established. For example, tuna oil primary emulsions prepared with $\beta$-lactoglobulin were less stable than secondary emulsions with an additional layer of $\iota$-carrageenan against aggregation and ionic and thermal stresses (Gu, Regnier, & McClements, 2005). Similar observations were reported when lecithin was used to prepare tuna oil emulsions that were subsequently coated with cationic chitosan (Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005). The thicker interface in secondary emulsions than that in primary emulsions provides better stability against droplet aggregation and coalescence, as well as elasticity against deformation (David Julian McClements, 2004).

### *2.4.3. Zeta potential of reconstituted secondary emulsions*

Zeta potential of reconstituted secondary emulsions after removing free molecules is compared to that of protein and pectin solutions in Figure 2-2. The respective pI of WPI and NaCas is about 4.2 ± 0.3 (Axel Benichou, Aserin, Lutz, & Garti, 2007) and 4.6 (Avena-Bustillos & Krochta, 1993), which agreed with the pH corresponding to zero zeta potential of protein solutions (Figure 2-2). For pectin, the magnitude of negative zeta potential decreased with

decreasing pH and was less than -5 mV at pH 2.0, which is caused by the protonation of carboxylate groups with pKa of 3.0 (Leenheer, Wershaw, & Reddy, 1995; Lutz, et al., 2009). At pH 6.0 and 7.0, both proteins and pectin were negatively charged and had similar magnitudes. Therefore, the zeta-potential data alone are insufficient to verify the adsorption or desorption of pectin on/from droplets around neutral acidity. At pH 3-5 (≤ pI of proteins), the zeta potential of droplets was similar to that of pectin at the same pH, which suggest the successful deposition of pectin on the droplets coated by proteins. At pH 2, the droplets had positive zeta-potential, agreeing with the higher magnitude of (positive) zeta-potential of proteins than that of (negatively-charged) pectin. It was also discussed in a previous study that pectin molecules cannot completely cover the droplet surface with proteins at pH 2 because of the weak electrostatic attraction between pectin and proteins at pH below the pKa of carboxylate group (Girard, Turgeon, & Gauthier, 2003).

### *2.4.4. Optical micrographs of reconstituted secondary emulsions*

Figure 2-3 shows optical microscopy images of reconstituted secondary emulsions at pH 2.0-7.0. At pH 2.0, irregular and/or aggregated structures were observed for reconstituted emulsions prepared with both WPI and NaCas. This observation was in accordance with the low zeta potential magnitude of droplets at pH 2.0 (Figure 2-2), which suggests the weak electrostatic repulsion led to the aggregation of droplets (de Kruif, Weinbreck, & de Vries, 2004). In addition, the weak ionization of carboxylate groups at pH 2.0 can result in some dissociation of pectin from droplet surface, and the free pectin molecules can cause depletion flocculation of droplets.

At pH 3.0 and 4.0, droplets were discrete and spherical, indicating the inter-particle interactions were effective in preventing droplet aggregation. Normally, droplets coated by NaCas will aggregate at pH close to its pI, i.e. around 4.0 - 5.0, and the aggregation of whey proteins at pH 4.0-5.0 is less likely than NaCas. The micrographs suggest that the deposition of pectin on droplet surface prevented the aggregation at pH 3-5 (Douglas G. Dalgleish, 1997). At pH 6.0 and 7.0, flocculated droplets were observed for WPI treatments, while fewer aggregates were observed for NaCas treatments. At an acidity above the protein pI, both proteins and pectin are negatively charged, and pectin is expected to be detached from the droplets. It however is possible to have the adsorption of pectin on cationic surface amino acid residues of overall negatively charged proteins (Lutz, et al., 2009). If a considerable amount of free pectin is present in the aqueous phase, depletion flocculation of droplets is possible (Chanamai & McClements, 2001; E. Dickinson, 2003), even though electrostatic repulsion between droplets is strong. The extent of depletion flocculation is dependent on proteins emulsifying the oil phase. A study concluded that as little as 0.06% pectin could induce the depletion flocculation of WPI-stabilized emulsion droplets at pH 7.0 (Gancz, Alexander, & Corredig, 2006). Conversely, for NaCas-stabilized emulsions, pectin could bind to the surface of droplets even at pH above the pI of caseins (Eric Dickinson, Semenova, Antipova, & Pelan, 1998). At pH 6.0 and 7.0, the free pectin in reconstituted emulsions after centrifugation was quantified to be 0.57-0.75 mg/mL (Table 2-3), which was below the overall pectin concentration of 1.31 mg/mL. Furthermore, the free pectin concentration in WPI treatments was higher than that in NaCas treatments (Table 2-3) and may have induced depletion flocculation of droplets. Therefore, optical micrographs show the

significance of pH on the aggregation properties of reconstituted secondary emulsions and the significance of electrostatic interactions on droplet stability.

### *2.4.5. Glutamine release in reconstituted secondary emulsions*

Figure 2-4 demonstrates percentages of glutamine released from reconstituted secondary emulsions adjusted to pH 2-7 during storage for 2 weeks at 4 ℃. All treatments showed the gradual, but less than 25% release of glutamine in two weeks. The release properties as affected by pH seem to agree with emulsion structures discussed previously. At pH 2.0, pectin is weakly charged and its adsorption on protein-coated droplets is limited. At pH 3.0 and 4.0, double-layered interfaces are expected because of the opposite charges of proteins and pectin. While at pH 5-7, partial detachment of pectin from droplet surface is expected. The double-layered interfaces are more effective in stabilizing emulsion droplets than the corresponding treatments with a single protein layer and therefore are more effective in retaining glutamine during storage.

### *2.4.6. Release kinetics of glutamine during in vitro digestion*

The *in vitro* release kinetics of glutamine is shown in Figure 2-5 for the first 2 h during the simulated gastric digestion and the subsequent 4 h of simulated intestinal digestion. The cumulative release of glutamine from primary emulsions (50-70%) was higher than that from secondary emulsions (<50%) during the simulated gastric digestion, for both fresh (Figure 2-5A) or reconstituted (Figure 2-5B) samples. During the first 2-h incubation in the simulated gastric fluid, the enzymatic hydrolysis of protein layer by protease in the simulated gastric fluid likely is the major mechanism responsible for the release of glutamine (Sarkar, et al., 2009). The

digestion is expected to be slower if droplets are covered by pectin that is not digestible by enzymes in the simulated gastric and intestinal fluids (Guerin, Vuillemard, & Subirade, 2003). Furthermore, secondary emulsions prepared with NaCas had a lower percentage of glutamine release than those prepared with WPI during the 2-h incubation in the simulated gastric juice. This may have been caused by the difference in binding affinity of pectin to WPI and NaCas.

During the subsequent 4-h incubation in the simulated intestinal fluid, gradual release of glutamine was observed for both primary and secondary emulsions. Proteins and pectin are both negatively charged in the simulated intestinal fluid at pH 6.8, and the desorption of pectin exposes proteins to be available for digestion by proteases. Pancreatin also contains pancreatic lipase (Bauer, Jakob, & Mosenthin, 2005; Favé, Coste, & Armand, 2004) that hydrolyzes MF to accelerate the destabilization of droplets to release glutamine. After 6-h incubation, cumulative release of glutamine reached ~90% or higher and was similar for all treatments. The results indicate that the release kinetics of glutamine is dependent on the digestion of protein and MF layers encapsulating glutamine solid particles. Furthermore, the release of glutamine from the reconstituted emulsions was always lower than that from the fresh emulsions. This likely is due to incomplete hydration of spray-dried powder that lowers the accessibility to digestive enzymes (Madene, Jacquot, Scher, & Desobry, 2006; Yoshii, et al., 2001).

## 2.5. Conclusions

In this study, a simple but effective way to encapsulate glutamine solid particles in multiple-layered emulsions was achieved. With a given amount of MF and glutamine solid

particles, a large amount of protein used in emulsification enabled the higher efficiency of encapsulating glutamine and the better retention of water-soluble glutamine during the refrigerated storage. When compared with primary emulsions, secondary emulsions showed the improved encapsulation efficiency and enhanced retention of glutamine during storage. When spray-dried samples were reconstituted at pH 2.0-7.0, pH conditions enabling strong adsorption of pectin on droplet surface proteins corresponded to better retention of glutamine during storage. When emulsions were incubated in the simulated gastric juice, secondary emulsions retained glutamine better than primary emulsions. All treatments showed >90% release after the subsequent incubation for 4 h in the simulated intestinal fluid. Additionally, the reconstituted emulsions had a better retention of glutamine in simulated gastric fluid than fresh emulsions. Therefore, water-soluble bioactive compounds can be prepared as solid particles that can be encapsulated by lipids in forms of multiple-layered emulsions to minimize the release during storage and maximize the gradual release in intestines.

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## Appendix

Table 2-1. The arithmetic mean diameter ($d_{1,0}$) of primary and secondary emulsions prepared with WPI or NaCas at different milk fat suspension (O):aqueous solution (W) volume ratios.

| O:W volume ratio | $d_{1,0}$ (μm) in emulsions prepared with different emulsifiers | | | |
|---|---|---|---|---|
| | WPI | | NaCas | |
| | Primary | Secondary | Primary | Secondary |
| 1:5 | 9.11±0.03$^{c}$ | 8.34±0.19$^{d}$ | 12.17±0.21$^{a}$ | 10.83±0.08$^{b}$ |
| 1:7 | 6.95±0.11$^{ef}$ | 6.77±0.01$^{ef}$ | 11.56±0.30$^{a}$ | 10.66±0.09$^{b}$ |
| 1:9 | 6.40±0.02$^{f}$ | 5.68±0.01$^{g}$ | 7.24±0.34$^{e}$ | 7.02±0.06$^{ef}$ |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 2-2. Efficiency of encapsulating glutamine in primary and secondary emulsions prepared with different milk fat suspension (O):aqueous solution (W) volume ratios.

| Emulsifier | O:W volume ratio | Encapsulation efficiency (%) * | |
|---|---|---|---|
| | | Primary emulsion | Secondary emulsion |
| WPI | 1:5 | 92.02 ±3.11$^{ab}$ | 93.71±2.45$^{ab}$ |
| | 1:7 | 92.85±2.79$^{ab}$ | 93.83±2.41$^{ab}$ |
| | 1:9 | 94.64±1.13$^{ab}$ | 95.96±1.57$^{a}$ |
| NaCas | 1:5 | 76.81±9.04$^{c}$ | 88.17±4.45$^{b}$ |
| | 1:7 | 89.62±4.05$^{b}$ | 91.36±3.21$^{ab}$ |
| | 1:9 | 89.37±4.14$^{b}$ | 94.67±2.08$^{ab}$ |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 2-3. Free pectin in reconstituted secondary emulsions at pH 6.0 and 7.0.

| Emulsifier | pH | Free pectin (mg/mL)* |
|---|---|---|
| WPI | 6.0 | $0.69\pm0.02^{b}$ |
| | 7.0 | $0.75\pm0.01^{a}$ |
| NaCas | 6.0 | $0.57\pm0.01^{c}$ |
| | 7.0 | $0.59\pm0.03^{c}$ |

*Numbers are mean ± standard deviation from triple measurement. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Figure 2-1. Percentages of glutamine remaining encapsulated during 14-day storage of (A) primary and (B) secondary emulsions prepared with WPI, in comparison to (C) primary and (D) secondary emulsions prepared with NaCas. The glutamine suspended in milk fat was emulsified at 1:5, 1:7 and 1:9 volume ratios in the protein solutions when preparing primary emulsions.

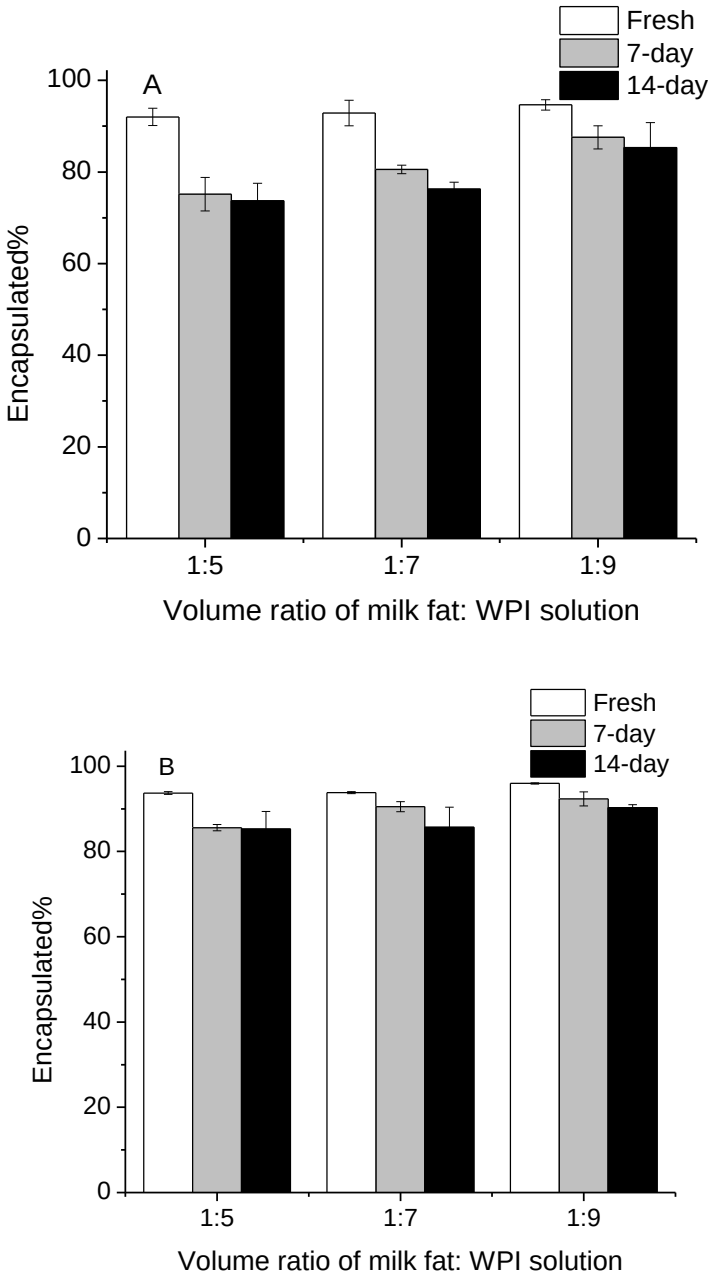
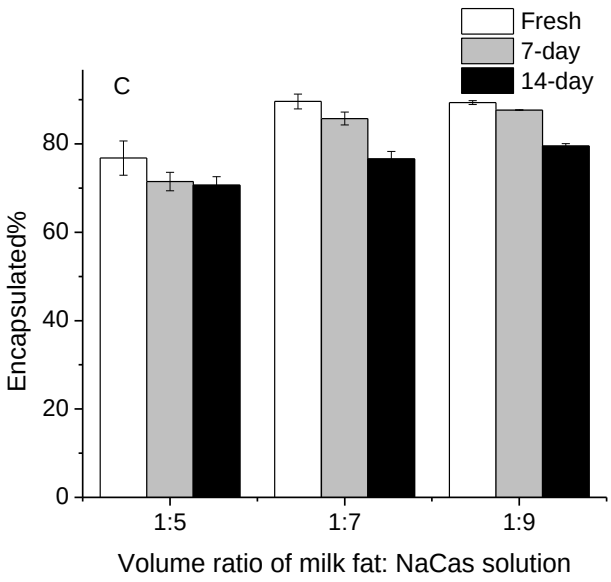



Figure 2-1. continued



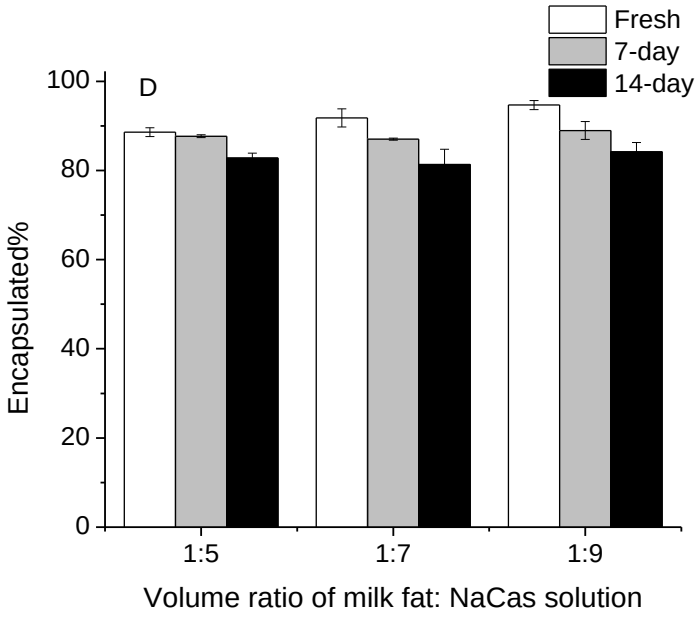




Figure 2-1. continued

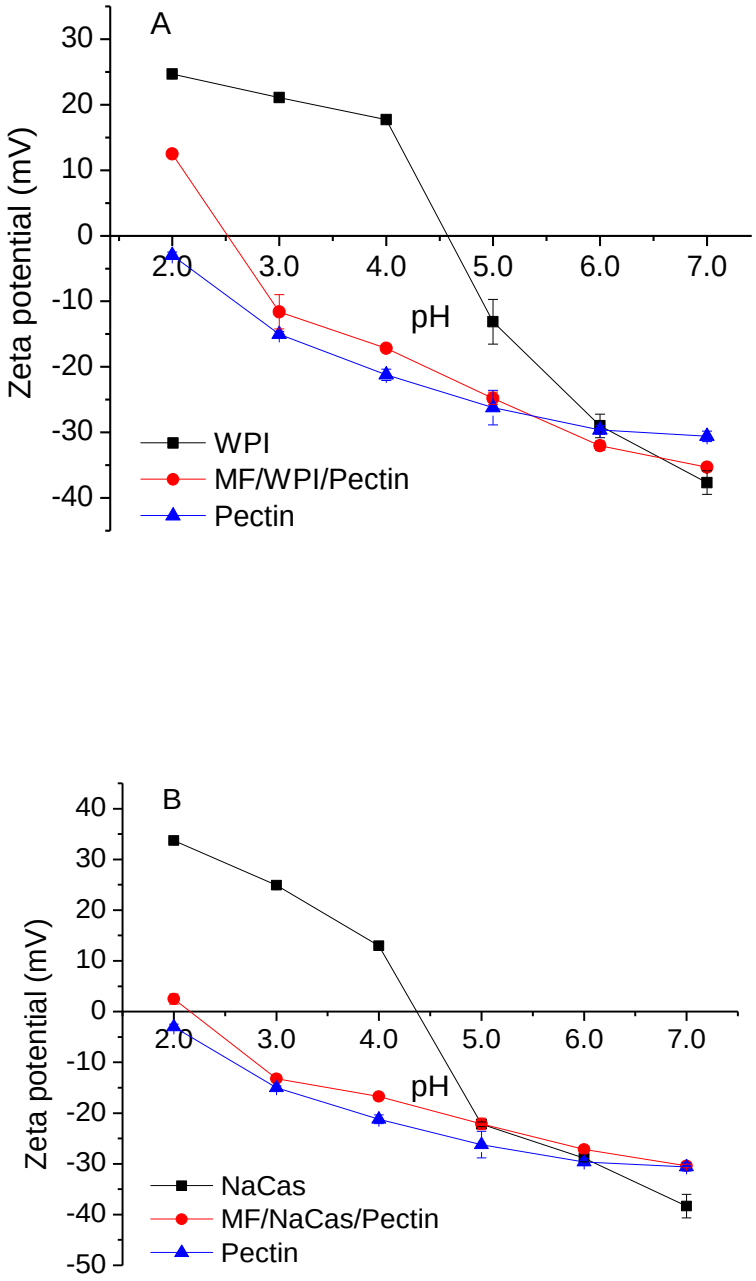



Figure 2-2. Zeta potentials of droplets in reconstituted secondary emulsions prepared with (A) WPI or (B) NaCas, pectin, and the corresponding protein at pH 2.0-7.0.

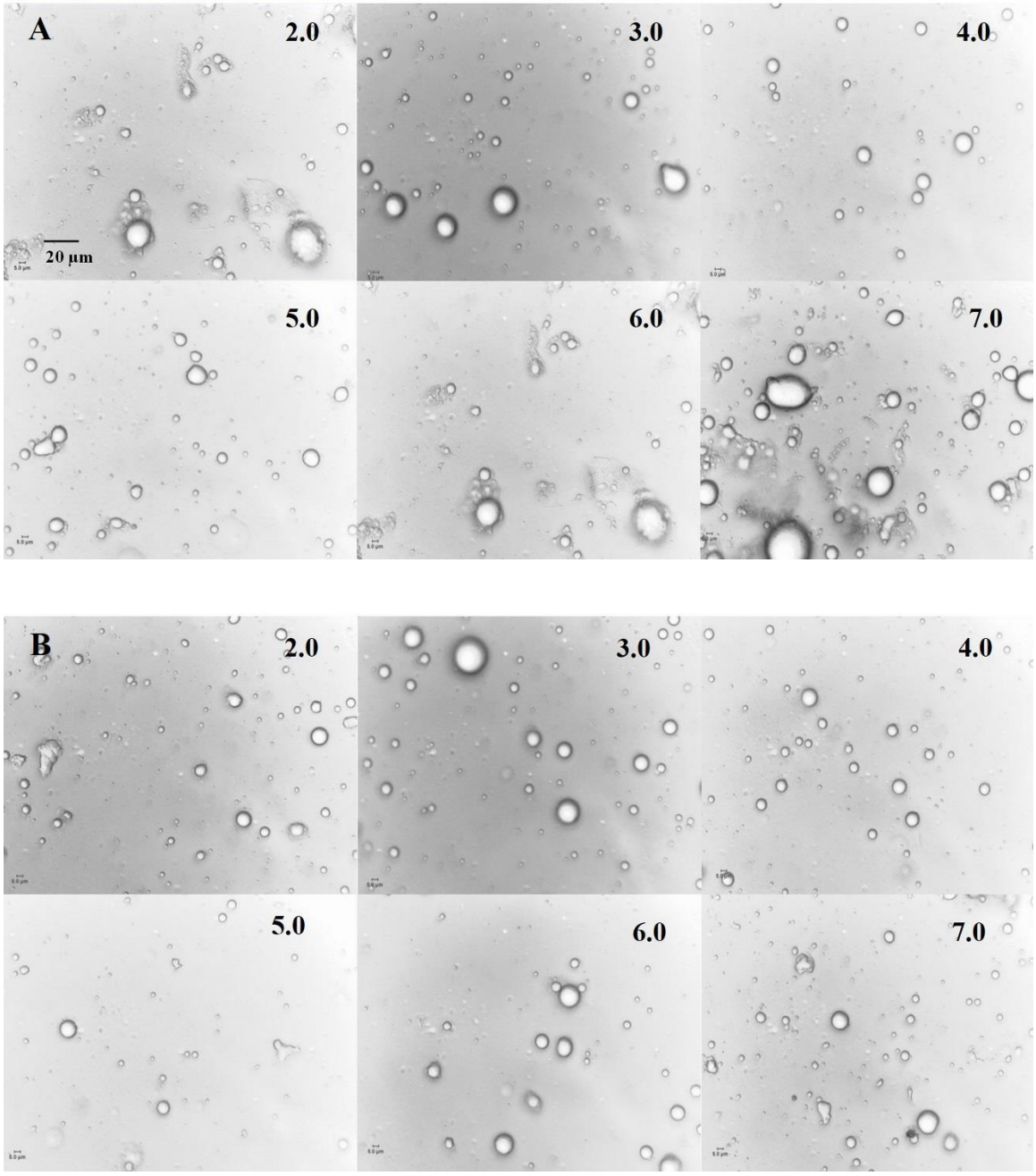



Figure 2-3. Optical micrographs of reconstituted secondary emulsions at pH 2.0-7.0, prepared with (A) WPI or (B) NaCas.

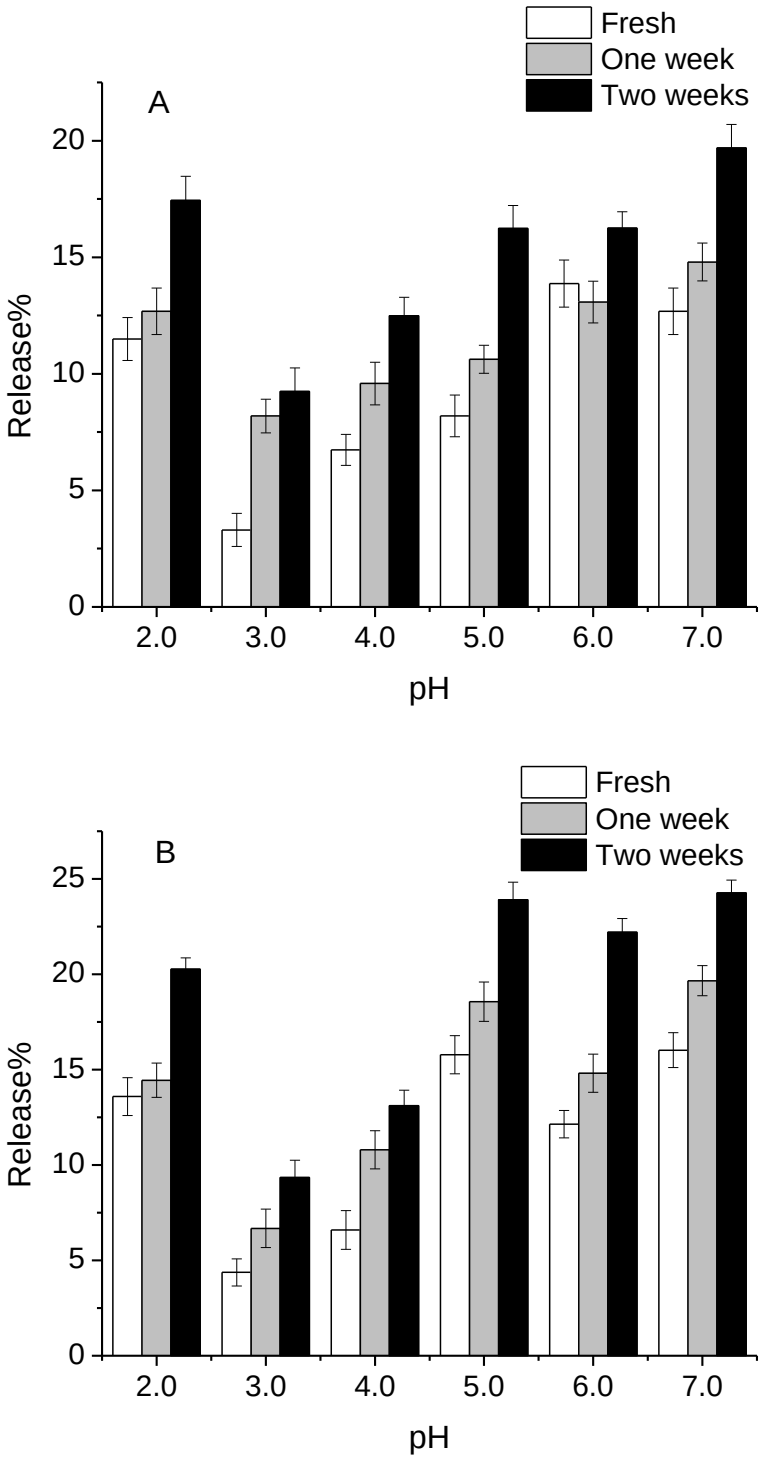



Figure 2-4. Release of glutamine in reconstituted secondary emulsions, prepared with (A) WPI or (B) NaCas and adjusted to pH 2.0-7.0, during two-week storage at 4 ℃.

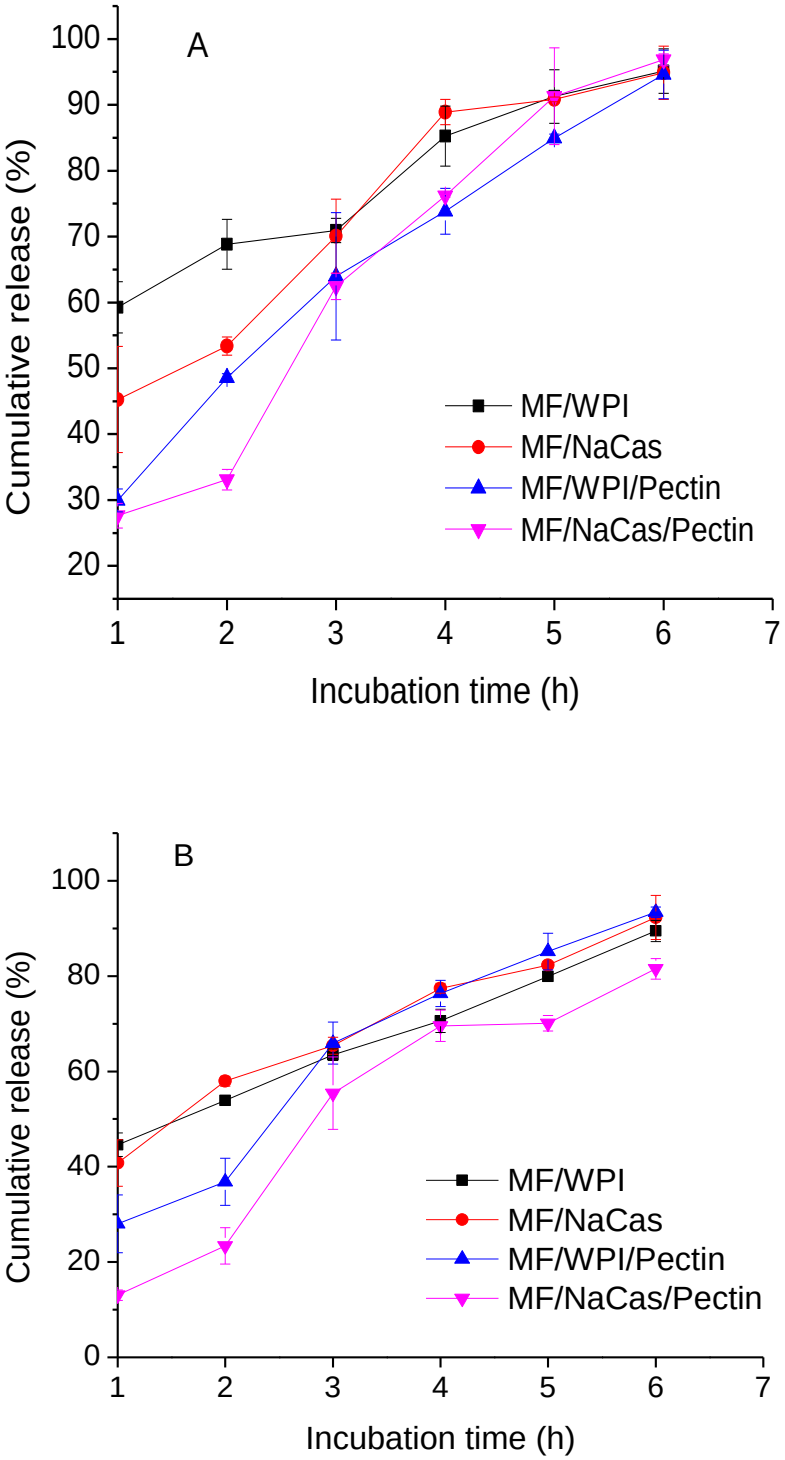



Figure 2-5. Release kinetics of glutamine from (A) fresh and (B) reconstituted emulsions during in vitro simulated gastric (first 2 h) and intestinal digestions (3-6 h).

# Chapter 3 . The increased viability of probiotic *Lactobacillus salivarius* NRRL B-30514 encapsulated in emulsions with multiple lipid-protein-pectin layers

A version of this chapter was originally published by Yun Zhang, Jun Lin and Qixin Zhong: Zhang, Y., Lin, J., & Zhong, Q. (2015). The increased viability of probiotic *Lactobacillus salivarius* NRRL B-30514 encapsulated in emulsions with multiple lipid-protein-pectin layers. *Food Research International*, *71*, 9-15.My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

## 3.1. Abstract

Probiotics have demonstrated various health benefits but have poor stability to sustain food processing and storage conditions, as well as after ingestion. Biopolymer beads are commonly studied to encapsulate probiotic cells to improve their stability, but the millimeter-dimension of these beads may not meet the quality requirement of food products. The aim of this study was to enhance the viability of *Lactobacillus salivarius* NRRL B-30514 by encapsulation in emulsion droplets with multiple lipid-protein-pectin layers. Spray-dried *L. salivarius* was suspended in melted anhydrous milk fat that was then emulsified in a neutral aqueous phase with whey protein isolate or sodium caseinate to prepare primary solid/oil/water (S/O/W) emulsions. Subsequently, pectin was electrostatically deposited onto the droplet surface at pH 3.0 to form secondary emulsions. The encapsulation efficiency was up to 90%. After 20-day storage at 4 ℃, the viable cell counts of bacteria in secondary emulsions at pH 3.0 and primary emulsions at 7.0 were 3 log higher than the respective free cell controls. After heating at 63 ℃ for 30 min, free *L. salivarius* was inactivated to be undetectable, while about 2.0 log CFU/mL was observed for primary (at pH 7.0) and secondary (at pH 3.0) emulsion treatments. Additionally, a 5 log-CFU/g-powder reduction was observed after spray drying free *L. salivarius*, while a 2 log CFU/g reduction was observed for emulsion treatments with capsules smaller than 20 μm. Furthermore, cross-linking the secondary emulsion with calcium enhanced the viability of *L. salivarius* after the simulated gastric and intestinal digestions. Therefore, the studied S/O/W emulsion systems may be used to improve the viability of probiotics during processing, storage, and gastrointestinal digestion.

**Keywords**: probiotics, viability, S/O/W emulsions, storage, digestion

### 3.2. Introduction

Probiotics are live microbial supplements that can benefit the microorganism balance of the host (Anal & Singh, 2007). Probiotics have significant effects on various human health issues, such as protecting the gastrointestinal tract by inhibiting the growth of undesirable bacteria and improving the immune system (Delcenserie et al., 2008). Probiotics may also have anti-tumor activities by producing bacteriocins and short-chain fatty acids and alleviate lactose intolerance by producing $\beta$-galactosidase (Delcenserie et al., 2008). Because of their potential health benefits, there are extensive interests in supplementing probiotics in various food products. The ultimate goal of probiotic supplementation is for these beneficial bacteria to survive and grow in the human body (Gilliland, 1989). This shall be assured by a sufficient population of viable probiotic bacteria before consumption and the ability of probiotics to survive physiological conditions in the gastrointestinal tract (Kailasapathy, 2002). For example, yogurt is the most popular food matrix suggested to include at least $10^6$ CFU/mL probiotic bacteria (Kurmann & Robinson, 1991). However, there are numerous factors affecting the survival of probiotic bacteria in food products, such as pH, temperature, and water activity (Krasaekoopt, Bhandari, & Deeth, 2003). Gastric acidity and inhibitory actions of bile salts are major obstacles for the survival of probiotic bacteria after ingestion (Bezkorovainy, 2001). Therefore, novel approaches are demanded to improve the viability of probiotics *in vitro* and *in vivo*.

Encapsulation is a group of technologies widely used in the food industry to improve product quality, such as extending shelf-life, protecting bioactive compounds from degradation,

and controlling the release of selected compounds. Various generally-recognized-as-safe (GRAS) biopolymers are studied as encapsulation materials, including gelatin, pectin, and alginate, to improve the viability and shelf-life of probiotic bacteria (Anal & Singh, 2007). Biopolymer beads produced by extrusion and in water-in-oil (W/O) emulsion template are commonly applied to encapsulate probiotics (Krasaekoopt et al., 2003). Extrusion is the most studied method due to its low cost and simplicity. This is studied by extruding a cell suspension with a gelling polymer such as alginate into a solution containing gelling agents such as $CaCl_2$ to create beads with immobilized probiotic cells (Cachon & Divies, 1993). However, the big dimension of beads (2-5 mm) limits their application in food products due to the sensory defect (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). In the W/O emulsion approach, carrageenan, gelatin and locust bean gum are commonly dissolved as supporting materials in cell suspensions that are emulsified in a continuous oil phase, followed by cross-linking within the emulsified aqueous droplets using an appropriate mechanism. (Krasaekoopt et al., 2003). The gel particles as prepared have a dimension of around 25 μm -2 mm and can be harvested from the oil phase by filtration or centrifugation. Compared to the extrusion technique, the emulsion template technique has the advantages of scalability and smaller beads (Manojlović, Nedović, Kailasapathy, & Zuidam, 2010). However, these beads can also potentially cause the sandy texture that results from particulates bigger than about 10 μm for dairy products (Walstra, Walstra, Wouters, & Geurts, 2005).

Recently, we studied multiple-layered solid/oil/water (S/O/W) emulsions to encapsulate spray-dried microparticles of water-soluble glutamine (Zhang & Zhong, 2015). The inclusion of

glutamine in the lipid layer limited its release to the continuous phase during prolonged storage, and the protein/pectin layers on droplet surfaces controlled its release during the simulated gastrointestinal digestion. Because probiotic cells have a dimension of about 1-4 μm (Burgain, Gaiani, Linder, & Scher, 2011) that is comparable to spray-dried glutamine particles, we hypothesize the multiple-layered S/O/W emulsions can be used to encapsulate probiotic cells. Unlike biopolymer beads with open and porous structures, the encapsulation of probiotics in multiple-layered lipid droplets may reduce the exchange with the continuous aqueous phase when incorporated in food products such as yogurt and therefore enhance the viability during processing and storage. The multiple layers of biopolymers on droplets may also reduce the acidity stress and inactivation by compounds such as bile salts during digestion and therefore improve the possibility to arrive and colonize in the lower intestinal tract.

The specific objective of the present work was to study the survival of spray-dried probiotic *Lactobacillus salivarius* NRRL B-30514 after encapsulation in S/O/W emulsions, during refrigerated storage, after thermal treatments, after dehydration, and after simulated gastrointestinal digestions. The spray-dried cells suspended in lipids were emulsified in the neutral aqueous phase with whey protein isolate (WPI) or sodium caseinate (NaCas) as the primary S/O/W emulsions. The primary emulsions were adjusted to pH 3.0 to electrostatically deposit an additional layer of pectin on droplets to form double-layered emulsions as secondary emulsions. *L. salivarius* NRRL B-30514 was chosen as a model probiotic strain because of its published genome (Wang et al., 2012) and its identified features of being probiotic bacteria (Messaoudi et al., 2013)..

## 3.3. Materials and methods

### *3.3.1. Materials*

WPI (93.4% protein, dry basis) was a kind gift from Hilmar Ingredients (Hilmar, CA, USA). NaCas (93% protein, dry basis) was purchased from American Casein Co. (Burlington, NJ, USA). Pectin from citrus peel with a galacturonic acid content no less than 74.0%, dry basis, was procured from Sigma Aldrich Corp. (St. Louis, MO, USA). Anhydrous milk fat (MF) was provided by Land O'Lakes, Inc. (Arden Hills, MN, USA). Other chemicals were from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

### *3.3.2. Preparation of bacteria for encapsulation*

The frozen stock culture (1 mL) of *L. salivarius* NRRL B-30514 in deMan, Rogosa and Sharpe (MRS) with 33.33% v/v glycerol was inoculated in 280 mL MRS broth and incubated at 37 °C for 18 h under anaerobic conditions. Anaerobic conditions were achieved using an anaerobic jar and anaerobe container system sachets with indicator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells were harvested by centrifugation at 3000 *g* for 30 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT, USA) at 4 °C and washed twice with a phosphate buffer solution (pH 7.4), with centrifugation at above conditions in each step. The washed cells were suspended at about $10^8$ CFU/mL in 280 mL of skim milk supplemented with 1% sucrose (Gardiner et al., 2000) or 280 mL of 0.1% peptone solution. The suspensions were then spray dried (model B290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) at a

pump rate setting of 10%. The inlet air temperature was 165 °C, and the outlet temperature was 90 °C. The respective spray-dried samples prepared from skim milk and peptone suspensions are referred as smCells and pCells hereafter. The powdered samples were stored at –20 °C before further experiments.

### *3.3.3. Encapsulation of spray-dried cells*

All glassware, centrifuge tubes, pipet tips and solutions used in this study were sterilized at 121 ℃ for 15 min. The emulsion preparation followed our previous study (Zhang & Zhong, 2015). Briefly, a S/O suspension was prepared by suspending spray-dried powder at 6% w/v in melted MF with stirring at 40 °C, corresponding to a cell population of about 8 log CFU/mL. The S/O suspension was emulsified in a neutral aqueous solution with 1.0% w/v WPI or NaCas to prepare primary S/O/W emulsions by homogenizing at 12,000 rpm for 2 min using a Cyclone I.Q. microprocessor homogenizer (VirTis Co., Gardiner, NY, USA). Subsequently, the primary emulsion was mixed with an equal volume of a 1.0% w/v pectin solution prepared separately, and the mixture pH was then adjusted to 3.0 using 1.0 M HCl to prepare secondary emulsions. The prepared emulsions were stored at 4 °C prior to analyses. Separate emulsions were spray-dried at above conditions, and the powder was kept at -20 °C before further analysis.

### *3.3.4. Cross-linking of pectin in secondary emulsions*

The secondary emulsions prepared as above at pH 3.0 was mixed with a 1% w/v calcium chloride solution at a volume ratio of 5:1. After stirring at room temperature (21 °C) for 2 h, the

suspension was either stored at 4 °C or spray-dried as above. The collected powder was stored at -20 °C before *in vitro* digestion experiments.

### *3.3.5. Enumeration of bacteria*

The enumeration of *L. salivarius* was conducted using the pour plate method. Unencapsulated *L. salivarius* was determined by serially diluting emulsions in 0.1% peptone solution directly and plating on MRS agar. The plates were incubated at 37 °C for 48 h in anaerobic conditions before enumeration. For smCell and pCell, 0.1 g spray-dried powder was suspended in 10 mL of 1 M sodium phosphate buffer (pH 7.4) and serially diluted, followed by incubation and enumeration as above.

To determine the viable cell count of encapsulated *L. salivarius*, the cells were released before enumeration. For fresh primary and secondary emulsions, polyoxyethylene (20) sorbitan monolaurate (Tween® 20) was used to displace proteins on droplet surface to release bacteria (Dickinson & Tanai, 1992). Tween® 20 was added at 0.75% w/v in 10 mL emulsion adjusted to pH 7.0, and the mixture was agitated by vortexing for 40 min at 21 °C. These displacement conditions were identified in preliminary experiments that showed the complete removal of proteins from oil droplets. Bacterial cells were collected by centrifugation at 10,000 *g* for 1 min using a MiniSpin Plus centrifuge (Eppendorf, Inc. Hauppauge, NY, USA), followed by enumeration using the above plating procedures. For spray-dried samples, the powdered samples were reconstituted in a pH 7.0 phosphate buffer for both primary and secondary emulsion treatments, followed by adding Tween® 20 and enumeration.

To determine the viable cells in the powder sample produced from secondary emulsions after calcium cross-linking, 0.5 g of the powdered sample was reconstituted in 10 mL buffer containing 0.05 mM of $NaH_2PO_4$ adjusted to pH 8.0 to chelate calcium and weaken the pectin coating to release bacteria (Annan, Borza, & Hansen, 2008). After incubation at room temperature for 30 min with gentle shaking, the suspension was centrifuged at 12,000 *g* for 2 min using a MiniSpin Plus centrifuge (Eppendorf, Inc. Hauppauge, NY). The precipitate was resuspended in 10 mL pH 7.0 phosphate buffer, followed by adding Tween® 20 and other procedures as presented above.

### *3.3.6. Encapsulation efficiency (EE)*

To measure the EE defined in Equation 1, the population of unencapsulated live cells was determined by direct enumeration of emulsions. The total population of live cells after encapsulation was determined after addition of Tween® 20 and enumeration using the method in Section 2.5. The total population of live cells used to prepare emulsions was determined by centrifuging the S/O suspension, resuspending the harvested cells in phosphate buffer (pH 7.4) at the same volume as the emulsion treatments, and enumeration as in Section 2.5.

$$\text{EE}\,(\%) = \left( \frac{\text{Total live cells after emulsification - Unencapsulated live cells, CFU/mL}}{\text{Total live cells before emulsification, CFU/mL}} \right) \times 100\% \qquad (1)$$

### *3.3.7. Scanning electron microscopy (SEM)*

The spray-dried smCells and primary and secondary emulsions prepared with WPI were imaged using a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany).

Before imaging, spray-dried samples were glued onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of about 5 nm in thickness.

### *3.3.8. Confocal laser scanning microscopy (CLSM)*

To study the structure of emulsion droplets, a Leica TCS SP2 microscope (Leica Microsystems, Heidelberg GmbH, Germany) was used. The microscope was equipped with an Ar ion laser with an excitation wavelength of 488 nm and two HeNe lasers with excitation wavelengths of 543 and 633 nm. To prepare emulsions for CLSM, 0.1 g WPI was dissolved in 10 mL of sodium bicarbonate buffer (pH 9.0), 4 mg fluorescent isothiocyanate (FITC) was added to WPI solution, and the mixture was gently stirred for 1 h at 21 °C (Twining, 1984) before adjusting pH to prepare primary emulsions as above. To stain MF, 0.001 g Nile red was dissolved in 10 mL propanol glycerol with gentle stirring for 1 h (Moschakis, Murray, & Dickinson, 2005), and 2 mL of the melted MF was mixed with 100 μL of the Nile red solution. The mixture was stirred for 1 h at 40 °C before preparation of primary emulsions as above. In order to remove free FITC, 1 mL of the emulsion was centrifuged at 12,000 *g* for 2 min, and the precipitate was resuspened in 1 mL sodium bicarbonate buffer (pH 9.0) for imaging.

To verify the viability of *L. salivarius* after encapsulation, cells and MF were labelled before preparing the primary emulsion. Cells were labeled using a BD Cell Viability staining kit (BD Biosciences, San Jose, CA, USA) following the manual, while MF was stained with Nile red as above. Cells were released from the primary emulsion with the above method used in

bacterial enumeration. The harvested *L. salivarius* cells were suspended in phosphate buffer (pH 7.4) before imaging.

### *3.3.9. Viability of L. salivarius during storage*

Primary emulsions at pH 7.0 and secondary emulsions at pH 3.0 with and without calcium were stored at 4 ℃. The smCell and pCell were used as controls by suspending 0.5 g of spray-dried powder in 10 mL phosphate buffer adjusted to pH 7.0 and 3.0. Emulsion or control samples were enumerated for the survival cell counts using the above methods after storage at 4 °C for 0, 10, and 20 days.

### *3.3.10. Viability of L. salivarius after heat treatment*

To measure the ability of encapsulated bacteria surviving thermal pasteurization, 10 mL of the primary emulsions, secondary emulsions with and without calcium, or free cell controls were heated at 63 °C for 30 min or 72 °C for 15 s in a water bath. These thermal treatment conditions are the requirements for pasteurization of Grade A milk (FDA, 2011). The temperature in the vials was verified using a thermal couple. After heat treatment and cooling in a room temperature water bath, survival cell counts were enumerated using the method in Section 2.5. Control samples with smCell were prepared by suspending 0.5 g spray-dried powder in 10 mL phosphate buffer adjusted to pH 7.0 or pH 3.0. Ten mL of a commercial yogurt (Dannon Activia®, The Dannon Company, Inc., White Plains, NY, USA) was also treated as above as another control.

### *3.3.11. Impacts of spray drying on the viability of L. salivarius*

Primary and secondary emulsions with and without calcium cross-linking were spray-dried using the conditions detailed previously. The smCell and pCell before and after spray-drying were compared as controls. The enumeration protocol was detailed in Section 3.3.5.

### *3.3.12. Viability of L. salivarius after in vitro digestions*

Spay-dried powder samples of the primary emulsion and secondary emulsions with and without calcium cross-linking were evaluated for their survivability after *in vitro* digestions. The smCell powder was used as a control. The simulated gastric and intestinal fluids were prepared using a literature method (Chávarri et al., 2010; Oomen et al., 2002) with modification. The simulated gastric fluid contained 3 g/L of pepsin and 8.5 g/L sodium chloride and was adjusted to pH 1.8 with 1.0 M HCl. The simulated intestinal fluid had 10 g/L of pancreatin, 8.5 g/L of sodium chloride, 3 g/L of bile salt, and 10 g/L of trypsin, and the pH was adjusted to 6.5 using 1.0 M HCl or NaOH.

To perform *in vitro* digestions, 0.1 g of spray-dried powder was mixed with 15 mL of the simulated gastric fluid and incubated at 37 °C in a shaking water bath operating at 150 rpm (New Brunswick Scientific Co., Edison, NJ, USA) for up to 2 h. During incubation for 1 and 2 h, 1 mL of the sample was withdrawn, and survival cells were enumerated as above. The sample after the treatment by the simulated gastric fluid was dissolved with bile salts, trypsin, and pancreatin directly to the aforementioned concentrations, and the pH was adjusted to 6.5. The mixture was

incubated at 37 °C in the above shaking water bath for 1, 2, 3, and 4 h before withdrawing 1 mL mixture to enumerate survival cell counts as described in Section 3.3.5.

#### *3.3.13. Statistical analysis*

Means and standard deviations were calculated from three independent emulsion replicates. A one-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL). The least-significant-difference (LSD) test was used to determine the significant differences of mean at a *P* level of 0.05.

## 3.4. Results and discussion

### *3.4.1. Encapsulation efficiency (EE)*

Table 3-1 shows the efficiency of encapsulating *L. salivarius* in emulsions. The emulsifier type (WPI or NaCas) did not affect the EE significantly ($P > 0.05$) for both primary and secondary emulsion treatments. However, the EE of secondary emulsion treatments was significantly higher than that of primary emulsions ($P < 0.05$). The highest EE of about 90% was observed for the secondary emulsion prepared with WPI.

### *3.4.2. Viability of L. salivarius during storage*

The viability of *L. salivarius* during storage at 4 °C is shown in Table 3-2. The population of *L. salivarius* in the pCell control reduced from 6.9 to 3.2 log CFU/mL after 20-day storage at pH 7.0, while *L. salivarius* in the smCell control showed a reduction of 3.3 log CFU/mL. At pH 3.0, there was a 5.5-log CFU/mL reduction for the pCell control and a 3.9-log CFU/mL reduction for the smCell control. The smCell had a better viability than the pCell

possibly because of the presence of lactose from skim milk. It was demonstrated that metabolizable saccharides such as glucose enhanced the survival of *L. rhamnosus* GG in acidic environments, because the metabolism of glucose provided ATP to ATPase through glycolysis (Corcoran, Stanton, Fitzgerald, & Ross, 2005). Studies also showed that disaccharides can protect bacteria by displacing water molecules nearby the cell membrane (Chávez & Ledeboer, 2007).

The viability of *L. salivarius* was significantly improved after encapsulation in the primary emulsions or secondary emulsions with and without calcium cross-linking (Table 3-2). The reduction of viable cell counts for *L. salivarius* encapsulated in the two primary emulsions was both 2.5 log CFU/mL after 20-day storage at pH 7.0, while the respective reduction at pH 3.0 was 1.8 and 1.5 log CFU/mL for secondary emulsions without and with calcium. Because the viability of *L. salivarius* was lower during storage at pH 3.0 than at pH 7.0 (Table 3-2), the higher viability of *L. salivarius* encapsulated in secondary emulsions than in primary emulsions should have resulted from differences in the emulsion structure. The thicker interface of secondary emulsions than that of primary emulsions provides a better stability against droplet aggregation and coalescence, as well as elasticity against deformation (McClements, 2004).

The improved storage viability of probiotics after encapsulation has been reported frequently. In a study, *Bifidobacterium breve* R070 was suspended in pre-heated whey protein dispersion and spray-dried, and the spray-dried cells in yogurt showed 2.6 log CFU/g yogurt higher than the free cells after 28-day storage at 4 °C (Picot & Lacroix, 2004). In another study, *Lactobacillus* F19 and *Bifidobacterium* Bb12 were encapsulated in casein-based microcapsules

by enzymatic gelation followed by freeze-drying, and the encapsulation improved the survival rate of powdered cells during storage at 4 or 25 °C and 11 or 33% relative humidity for up to 90 days (Heidebach, Först, & Kulozik, 2010). Similarly, spray-drying *Bifidobacterium infantis* strain suspended in an O/W emulsion showed a 5 log enhancement than the control after two-week storage of the powdered sample at 25 °C and 55% relative humidity (Crittenden, Weerakkody, Sanguansri, & Augustin, 2006).

### *3.4.3. Dimension of spray-dried capsules studied by SEM*

Figure 3-1 shows SEM images of smCells and spray-dried powder with *L. salivarius* encapsulated in the primary emulsion prepared with WPI and the secondary emulsion prepared with WPI and pectin. Particles in smCells were smaller than 5 μm, and the particle dimension increased after encapsulation in primary and secondary emulsions. Particles in the secondary emulsion treatment were the most heterogeneous. Particles in all treatments were mostly smaller than 10 μm, but a few particles of around 20 μm in diameter were also observed for the secondary emulsion treatment after scanning the entire sample (not shown). Therefore, the particles with encapsulated bacteria prepared in the present study were much smaller than the millimeter dimension prepared by the extrusion technique (Sohail et al., 2011) and appeared to meet the requirement of about 10 μm for use in dairy products (Walstra et al., 2005). The dimension of capsules with encapsulated bacteria in the present study is similar to that of powder particles (5-15 μm) spray-dried with probiotic bacteria and skim milk or gum acacia (Desmond,

Ross, O'callaghan, Fitzgerald, & Stanton, 2002). However, the dimension of spray-dried samples after rehydration was not reported by the authors.

### *3.4.4. Confocal laser scanning micrographs*

Figure 3-2 shows the CLMS micrographs of samples. In Figure 3-2A, the interfacial WPI (in green) on MF droplet (in red) confirmed the complete emulsification of MF. The encapsulation of *L. salivarius* (in green) in the primary emulsion was confirmed in Figure 3-2B. The stained *L. salivarius* cells separated from the primary emulsion using the procedures described in the method section are presented in Figure 3-2C. Both viable (in green) and dead (in red) cells were observed, while yellow ones are probably due to the overlap of viable and dead cells (due to the diffusion of cells during imaging). The viable cells verified the ability of spray-dried *L. salivarius* to survive the encapsulation conditions. Trace MF was also observed, indicating that the demulsification was complete and most MF was discarded after centrifugation. The CLSM results also suggest that the colony counts of *L. salivarius* should result from individual cells after plating on MRS agar and incubation.

### *3.4.5. Viability of L. salivarius after thermal treatment*

The populations of free and encapsulated *L. salivarius* before and after heating at 63 °C for 30 min are shown in Table 3-3. Free smCells were reduced to an undetectable level, which indicated the poor heat stability of *L. salivarius*. The complete reduction of probiotics in a commercial yogurt product was also observed. Similar results were reported in a study after heating *L. paracasei* NFBC 338 and *L. salivarius* UCC 118 strains at 59 °C for 4 min (Gardiner

et al., 2000). Comparing with free smCell, the viable cell count of encapsulated *L. salivarius* was higher after heating at 63 °C for 30 min (Table 3-3), and the survivability was significantly higher after encapsulation in the secondary emulsions than in the primary emulsions ($P < 0.05$). Further addition of calcium in the secondary emulsions did not significantly improve the cell viability after heating ($P > 0.05$). The populations of free and encapsulated *L. salivarius* before and after heating at 72 °C for 15 s are shown in Table 3-4. Overall, the results had the same trend as samples heated at 63 °C for 30 min (Table 3-3), but the survival of cells generally improved. The secondary emulsion with calcium had the highest ($P < 0.05$) viable cell counts after heating, with about 3.3-log CFU/mL reduction. Therefore, high-temperature-short-time pasteurization is preferred for pasteurizing products containing encapsulated probiotics.

During heating, the death of cells is attributed to the destroyed higher-ordered structures of proteins and nucleic acids in cells and the collapsed linkages between monomeric units (Fritzen-Freire, Prudêncio, Pinto, Muñoz, & Amboni, 2013). Encapsulation can provide a physical barrier against harsh environmental conditions (Kailasapathy, 2002), which may be the case of the present study (Tables 3-3 and 3-4). In a study, the survival of *L. casei* NCDC-298 microencapsulated in 2%, 3% or 4% calcium alginate increased by 1-2, 3-4, and 3-4 log from the free cell control after heating at 55, 60 or 65 °C for 20 min (Mandal, Puniya, & Singh, 2006). The authors speculated that the improvement in heat stability after encapsulation resulted from the lowered water diffusion rate in alginate beads with a higher concentration of biopolymers (Mandal et al., 2006). Additionally, the reduction of viable cells after heating is not only

attributed to thermal treatment conditions but also the disappearance of water molecules bound on the cell surface (Daemen, 1981). In the present study, coating MF on cells (Figure 3-2B) likely can maintain the cell structure better than cells suspended in an aqueous continuous phase, thereby improving the thermal stability. The extent of improvements in our study is different from the above references, which can be attributed to differences in thermal treatment conditions, bacterial strains, and the environment surrounding the cells.

### *3.4.6. Viability of L. salivarius after spray drying*

Table 3-5 summarizes the viability of free and encapsulated *L. salivarius* after spray drying. The reduction of viable cell counts of bacteria suspended in the peptone solution (5.63 log CFU/g, in preparation of pCell) was the most significant, which may be attributed to the poor thermal tolerance of *L. salivarius* (Table 3-3). The reduction of viable cell counts in preparing smCell (3.54 log CFU/g) was lower than that of pCell. Heat inactivation is the major reason for the reduction of viable cells after spray drying, and the decreased survival rate of *L. salivarius* in 20%w/v reconstituted skim milk was reported at the increased outlet temperature during spray drying (Gardiner et al., 2000). Spray drying used to prepare smCell can therefore contribute to dead cells observed in Figure 3-2. As for the differences in log reductions when preparing smCell and pCell, it has been shown that sample composition and properties such as thermal conductivity and diffusivity can result in different log reductions after spray drying (Lian, Hsiao, & Chou, 2002) . The addition of gum acacia to the reconstituted skim milk was also reported to

protect the probiotic cells after spray drying (Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002).

For emulsion treatments, the viable cell counts after spray drying were significantly higher ($P < 0.05$) than free cell treatments (Table 3-5). The respective log reduction of primary emulsions was 1.01 and 0.97 log CFU/g for MF/WPI and MF/NaCas treatments. The secondary emulsions without calcium showed 0.44 and 0.63 log CFU/g reduction for MF/WPI/Pectin and MF/NaCas/Pectin treatments, respectively, and were significantly different ($P < 0.05$) from primary emulsions. Calcium cross-linking secondary emulsions did not additionally ($P \geq 0.05$) improve the viability of cells. Our findings agreed with studies using encapsulation to improve the survival of probiotics after spray drying. For example, microencapsulation of *Bifidobacteriumlactis* strain BI 01 and *L. acidophilus* strain LAC 4 in casein/pectin coacervates was effective in protecting the inactivation of bacteria after spray drying (Champagne, Raymond, Mondou, & Julien, 1995).

### *3.4.7. Survival of L. salivarius during in vitro digestion*

An important goal of probiotic supplementations is the survivability at gastrointestinal conditions. In the present study, viable cell counts of *L. salivarius* after the first 2 h incubation in the simulated gastric fluid and the following 4 h in the simulated intestinal fluid were determined for free cells and spray-dried emulsions prepared using WPI (Figure 3-3). During the first 2 h incubation in the simulated gastric fluid, the viable cell count gradually decreased for all samples, and the reduction was 0.55 log CFU/g for smCells, 0.44 log CFU/g for primary

emulsion, 0.34 log CFU/g for the secondary emulsion, and 0.16 log CFU/g for calcium cross-linked secondary emulsion, respectively. The results were consistent with another study that reported the survival of *L. salivarius* in the simulated gastric fluid due to the relatively high acid tolerance and the improvement of survivability after encapsulation (Chávarri et al., 2010). The higher reduction of viable cell counts in the primary emulsion than in the secondary emulsion can result from the protection by indigestible pectin coating capsule surface for the latter, therefore reducing the hydrolysis of protein layer and demulsification (Sarkar, Goh, Singh, & Singh, 2009). Among all the samples, *L. salivarius* encapsulated in the secondary emulsion with calcium had the smallest reduction of viable cell count, which can be explained by the additional strengthening of pectin layer after cross-linking by calcium ions (Sandberg, Ahderinne, Andersson, Hallgren, & Hultén, 1983).

In the subsequent 4-h incubation in the simulated intestinal fluid, both free and encapsulated *L. salivarius* showed the dramatic decrease in the viable cell counts, except the secondary emulsion cross-linked by calcium ions. This phenomenon is probably attributed to the inhibitory effects of bile salts on bacteria, as reported for *Clostridia*, *Bacteroides*, and *Lactobacillus* (Binder, Filburn, & Floch, 1975). For the case of smCells, the viable cell count reduced by 2 log after 2 h incubation and became undetectable after incubation for additional 2 h. For *L. salivarius* encapsulated in the primary emulsion, the viable cell count showed a 3.5-log CFU/g reduction after 3 h incubation and also reduced to below the detection limit after 4 h incubation. For *L. salivarius* encapsulated in the secondary emulsion, the viable cell count was 1.4 log CFU/g after 4 h incubation. These results concur with another study reporting that

encapsulation of probiotic bacteria can enhance the survival of cells in the simulated intestinal fluid (Chávarri et al., 2010). At the simulated intestinal conditions, both WPI and pectin are negatively charged, and the desorption of pectin exposes proteins for hydrolysis by proteases, which, facilitated by lipolysis by the lipase in pancreatin, demulsifies the capsules and exposes *L. salivarius* to bile salts (Bauer, Jakob, & Mosenthin, 2005). In the case of *L. salivarius* encapsulated in the calcium cross-linked secondary emulsion, there was 4.9 log CFU/g viable cells after 4 h incubation. This can also be explained by the cross-linking effect of calcium ions that prevent the detachment of pectin from droplet surfaces, thereby reducing the digestion of capsules (Sandberg et al., 1983). It is well-known that pectin can be degraded by pectinase produced by colonic microflora (Wong, Colombo, & Sonvico, 2011), and therefore the encapsulated probiotics can be released after surviving the digestion in the small intestine. This will require future in vivo verifications.

### 3.5. Conclusions

In summary, *L. salivarius* can be encapsulated in primary and secondary emulsions with an encapsulation efficiency of up to 90%. The encapsulation technology as proposed resulted in microcapsules that have promise for food applications without causing sandy texture. The encapsulation improved the storage and thermal stability of *L. salivarius*. The survivability of *L. salivarius* was better after heating at 72 °C for 15 s when compared to 65 °C for 30 min, especially for secondary emulsions. The encapsulated *L. salivarius* also had the significantly enhanced stability after spray drying. Lastly, encapsulation of the probiotic cells in the secondary

emulsion, particularly after cross-linking by calcium ions, greatly improved the survivability during the simulated gastric and intestinal digestions. If *L. salivarius* can colonize *in vivo*, the studied multiple-layered emulsions may be used to incorporate probiotics in various products and improve their viability during processing and storage and after ingestion.

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## Appendix

Table 3-1. Efficiencies of encapsulating *L. salivarius* in primary and secondary emulsions

| Samples | Encapsulation efficiency (%)* |
|---|---|
| Primary emulsions | |
| MF/WPI | $78.53 \pm 2.40^{b}$ |
| MF/NaCas | $77.78 \pm 2.64^{b}$ |
| Secondary emulsions | |
| MF/WPI/pectin | $91.51 \pm 4.32^{a}$ |
| MF/NaCas/pectin | $87.61 \pm 1.56^{a}$ |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 3-2. Viable cell counts of free and encapsulated *L. salivarius* during 20-day storage at 4 °C.

| Samples | Viable cell count (log CFU/mL) * | | |
|---|---|---|---|
| | Day 0 | Day 10 | Day 20 |
| Free cells [#] | | | |
| pCell (7.0) | $6.93\pm0.21^{ef}$ | $3.78\pm0.26^{o}$ | $3.17\pm0.22^{p}$ |
| pCell (3.0) | $6.93\pm0.21^{ef}$ | $2.87\pm0.22^{q}$ | $1.40\pm0.14^{r}$ |
| smCell (pH 7.0) | $7.19\pm0.02^{bcde}$ | $4.17\pm0.19^{m}$ | $3.90\pm0.08^{no}$ |
| smCell (pH 3.0) | $7.53\pm0.05^{a}$ | $3.95\pm0.07^{no}$ | $3.68\pm0.25^{o}$ |
| Primary emulsions at pH 7.0 | | | |
| MF/WPI | $7.36\pm0.11^{abc}$ | $6.02\pm0.09^{h}$ | $4.91\pm0.06^{l}$ |
| MF/NaCas | $7.27\pm0.05^{abcd}$ | $5.89\pm0.05^{h}$ | $4.76\pm0.07^{l}$ |
| Secondary emulsions at pH 3.0 | | | |
| MF/WPI/pectin | $7.43\pm0.09^{ab}$ | $6.91\pm0.07^{ef}$ | $5.62\pm0.02^{i}$ |
| MF/NaCas/pectin | $7.13\pm0.07^{cde}$ | $6.78\pm0.01^{f}$ | $5.52\pm0.06^{ij}$ |
| Secondary emulsions with 0.17%w/v $CaCl_2$ at pH 3.0 | | | |
| MF/WPI/pectin | $7.04\pm0.09^{def}$ | $6.50\pm0.02^{g}$ | $5.22\pm0.16^{k}$ |
| MF/NaCas/pectin | $6.79\pm0.01^{f}$ | $6.38\pm0.01^{g}$ | $5.33\pm0.06^{jk}$ |

* Numbers are mean ± standard deviation from triple measurements. Means with different superscript letters indicate significant differences ($P < 0.05$).

[#] The pCell and smCell controls were spray-dried powder, prepared from cells suspended in peptone or skim milk, reconstituted in phosphate buffers adjusted to pH 7.0 or 3.0.

Table 3-3. Viable cell counts of free and encapsulated L. salivarius before and after heating at 63 °C for 30 min.

| Samples | Viable cell count (log CFU/mL)* | |
|---|---|---|
| | Before heating | After heating |
| Yogurt | 8.01±0.03[a] | <1 |
| Free cells [#] | | |
| smCell (pH 7.0) | 6.67±0.11[b] | <1 |
| smCell (pH 3.0) | 6.53±0.05[b] | <1 |
| Primary emulsions at pH 7.0 | | |
| MF/WPI | 6.63±0.10[b] | 1.70±0.03[ef] |
| MF/NaCas | 6.42±0.02[b] | 1.50±0.05[f] |
| Secondary emulsions at pH 3.0 | | |
| MF/WPI/pectin | 6.55±0.07[b] | 2.08±0.05[cd] |
| MF/NaCas/pectin | 6.71±0.07[b] | 1.85±0.02[de] |
| Secondary emulsions with 0.17%w/v $CaCl_2$ at pH 3.0 | | |
| MF/WPI/pectin | 6.54±0.04[b] | 2.26±0.08[c] |
| MF/NaCas/pectin | 6.63±0.09[b] | 2.11±0.02[cd] |

*Numbers are mean ± standard deviation from triple measurements. Means with different superscript letters indicate significant differences ($P < 0.05$).

[#] The smCell controls were spray-dried powder, prepared from cells suspended in skim milk, reconstituted in phosphate buffers adjusted to pH 7.0 or 3.0.

Table 3-4. Viable cell counts of free and encapsulated *L. salivarius* before and after heating at 72 °C for 15 s.

| Samples | Viable cell count (log CFU/mL)* | |
|---|---|---|
| | Before heating | After heating |
| Yogurt | 7.90±0.02[a] | 1.18±0.23[h] |
| Free cells [#] | | |
| Skim milk (pH 7.0) | 6.68±0.04[b] | 1.10±0.05[h] |
| Skim milk (pH 3.0) | 6.70±0.03[b] | 1.11±0.14[h] |
| Primary emulsions at pH 7.0 | | |
| MF/WPI | 6.47±0.03[bc] | 2.18±0.03[g] |
| MF/NaCas | 6.42±0.16[bc] | 1.98±0.05[g] |
| Secondary emulsions at pH 3.0 | | |
| MF/WPI/pectin | 6.43±0.08[bc] | 2.73±0.04[ef] |
| MF/NaCas/pectin | 6.31±0.16[c] | 2.57±0.04[f] |
| Secondary emulsions with 0.17%w/v $CaCl_2$ at pH 3.0 | | |
| MF/WPI/pectin | 6.33±0.02[c] | 3.06±0.07[d] |
| MF/NaCas/pectin | 6.31±0.14[c] | 3.00±0.05[de] |

*Numbers are mean ± standard deviation from triple measurements. Means with different superscript letters indicate significant differences ($P < 0.05$).

[#] The smCell controls were spray-dried powder, prepared from cells suspended in skim milk, reconstituted in phosphate buffers adjusted to pH 7.0 or 3.0.

Table 3-5. Reduction of viable cell counts of free and encapsulated *L. salivarius* after spray drying.

| Samples | Reduction of viable cell count (log CFU/g) * |
|---|---|
| Free cells | |
| Bacteria in peptone | 5.65±0.07[a] |
| Bacteria in skim milk | 3.50±0.01[b] |
| Primary emulsions at pH 7.0 | |
| MF/WPI | 1.03±0.02[c] |
| MF/NaCas | 0.99±0.04[c] |
| Secondary emulsions at pH 3.0 | |
| MF/WPI/pectin | 0.43±0.06[e] |
| MF/NaCas/pectin | 0.63±0.02[d] |
| Secondary emulsions with 0.17%w/v $CaCl_2$ at pH 3.0 | |
| MF/WPI/pectin | 0.49±0.05[e] |
| MF/NaCas/pectin | 0.45±0.02[e] |

* Numbers are mean ± standard deviation from triple measurements. Means with different superscript letters indicate significant differences ($P < 0.05$).

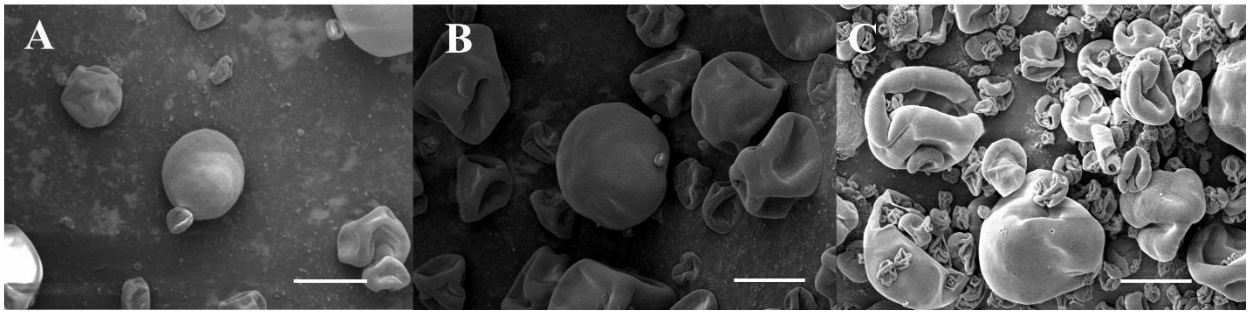


Figure 3-1. Scanning electron micrographs of spray-dried powder prepared from (A) cells suspended in skim milk, (B) cells encapsulated in the primary emulsion prepared with WPI, and (C) cell encapsulated in the secondary emulsion prepared with WPI and pectin. Bar =5 μm.

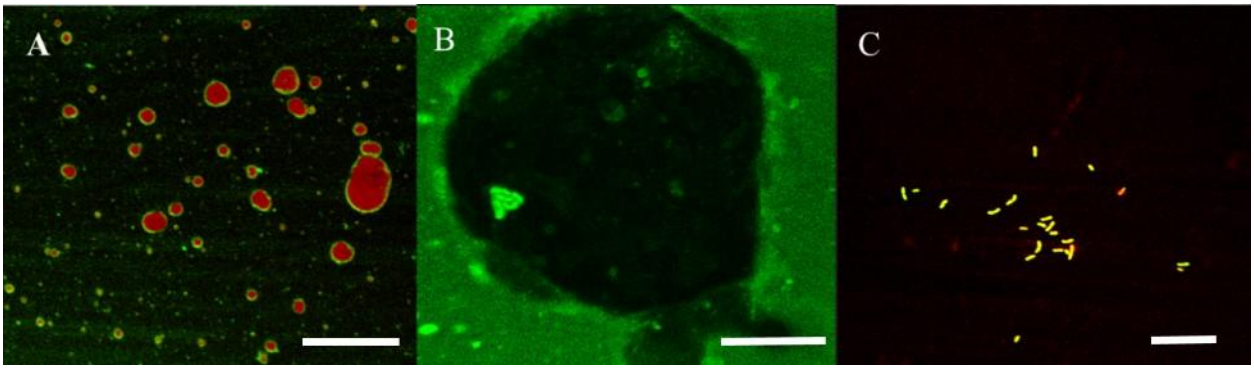


Figure 3-2. Confocal laser scanning micrographs of (A) primary emulsion prepared with WPI without bacteria, (B) *L. salivarius* encapsulated in the primary emulsion prepared with WPI, and (C) resuspended *L. salivarius* after centrifuging the primary emulsion mixed with Tween® 20 to displace surface whey proteins. Images in A and B are about 0.25 μm below the focal plane. Bar = 20 μm.

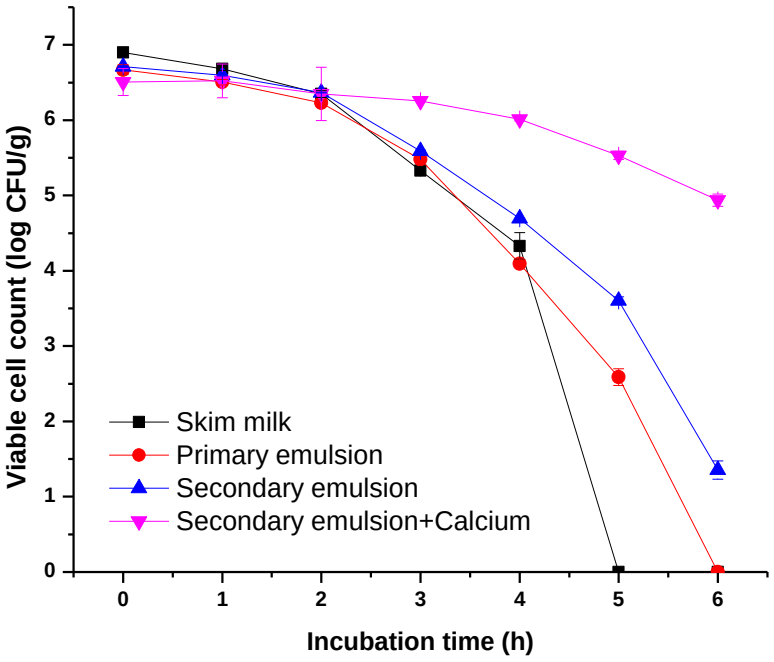


Figure 3-3. Viable cell counts of free (smCell) and encapsulated *L. salivarius* during in vitro simulated gastric (first 2 h) and intestinal (3-6 h) digestions. The primary emulsion was prepared with WPI and was adsorbed with pectin as the secondary emulsion that was also cross-linked with calcium ions. Error bars are standard deviations from triple measurements.

# Chapter 4 . S/O/W emulsions prepared with sugar beet pectin to enhance the viability of probiotic *Lactobacillus salivarius* NRRL B-30514

A version of this chapter was originally published by Yun Zhang, Jun Lin and Qixin Zhong: Zhang, Y., Lin, J., & Zhong, Q. (2016). S/O/W emulsions prepared with sugar beet pectin to enhance the viability of probiotic *Lactobacillus salivarius* NRRL B-30514. *Food Hydrocolloids*, *52*, 804-810. My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.

## 4.1. Abstract

Encapsulation is a common approach to improve the viability of probiotic bacteria against environmental stresses. However, conventional biopolymer beads have a millimeter dimension and can cause texture defects. The objective of this work was to study solid/oil/water emulsions as a delivery system by suspending spray-dried probiotic *Lactobacillus salivarius* NRRL B-30514 in soybean oil followed by emulsification in sugar beet pectin solution. The encapsulation efficiency was up to 87%, and the droplets were smaller than 17 μm. Encapsulation of *L. salivarius* in S/O/W emulsions improved the viability under different conditions, including 2-week storage at 4 °C, pasteurization at 63 °C for 30 min, and the *in vitro* gastric and intestinal digestions containing proteases and bile salt. Cross-linking sugar beet pectin on emulsion droplets by divalent calcium ions further improved the viability of *L. salivarius* against various environmental stresses, enabling the survival of a significant portion of viable *L. salivarius* following treatment with simulated gastrointestinal digestions. Findings from the present work suggest the possibility of the studied S/O/W emulsions to deliver probiotic bacteria in foods to improve their viability during processing, storage, and digestion.



**Keywords:** sugar beet pectin, S/O/W emulsion, probiotic bacteria, viability, storage, digestion

## 4.2. Introduction

Probiotics are viable microorganisms and substances that have positive physiological effects on intestinal microflora populations of the host (Guerra, Bernárdez, Méndez, Cachaldora, & Pastrana Castro, 2007). In recent years, probiotics also have drawn considerable attention as natural antibiotics in poultry products (Guerra, et al., 2007; Woraharn, Chaiyasut, Sirithunyalug, & Sirithunyalug, 2010). Common probiotics include *Lactobacillus* such as *L. salivarius*, *L. acidophilus*, and *L. casei*, *Bifidobacterium*, and yeasts such as *Saccharomycces cerevisiae* (AFRC, 1989). Lactic acid bacteria are major microorganisms in the gastrointestinal tract of humans and animals, and bacteriocins and organic acids produced by these bacteria have positive effects against infections (Twomey, Ross, Ryan, Meaney, & Hill, 2002). Studies also concluded that probiotics as feed supplements can improve the growth rate, general health, and ability to resist diseases of poultry (Ahmad, 2006; Guerra, et al., 2007).

However, there are several challenges in the application of probiotics as supplements, including the viability during processing and prolonged storage, and the survival in acidic conditions and digestive fluids especially the bile (Lin, Yu, Jang, & Tsen, 2007). Encapsulation is a commonly-studied technique to immobilize probiotics to enhance their survival rate in adverse environments. Entrapment of probiotics in calcium alginate beads is a widely used encapsulation approach. Encapsulation of nine *Bifidobacteria* strains in calcium alginate microspheres improved their survival ability in milk during refrigerated storage (Hansen, Allan-Wojtas, Jin, & Paulson, 2002). *L. acidophilus* CSCC 2400 was encapsulated in calcium alginate beads to improve the survival ability in simulated gastric conditions (Chandramouli,

Kailasapathy, Peiris, & Jones, 2004). *L. casei* NCDC-298 encapsulated in beads produced with different alginate concentrations had a better ability to survive low pH, high bile salt conditions, and during heating (Mandal, Puniya, & Singh, 2006). Pectin, chitosan and carrageenan are other biopolymers studied to encapsulate probiotics (Audet, Paquin, & Lacroix, 1991; Krasaekoopt, Bhandari, & Deeth, 2006; Sandoval-Castilla, Lobato-Calleros, García-Galindo, Alvarez-Ramírez, & Vernon-Carter, 2010).

Pectin is a heteropolysaccharide in the cell wall of higher plants (Thakur, Singh, Handa, & Rao, 1997). Commercial pectin ingredients are mainly extracted from citrus peels, sugar beet pulp, and apple pomace (Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003). Pectin is widely used as a food additive for gelling and stabilizing functions. High-ester pectin (degree of esterification, DE, >50%) from citrus and apple pomace can form gels at pH below 3.5 due to hydrogen bonding and hydrophobic interactions (Walkinshaw & Arnott, 1981), while low-ester pectin (DE < 50%) needs divalent cations such as calcium ions to form gels (Grant, Morris, Rees, Smith, & Thom, 1973). Sugar beet pectin (SBP) has poor gelling ability but has good emulsifying properties (Leroux, et al., 2003). The emulsification property of SBP is attributed to the protein segment glycated to polysaccharides and the acetyl group (Williams, et al., 2005).

Various methods have been studied to encapsulate probiotic bacteria, such as spray drying, emulsion, and extrusion (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). The extrusion method is the oldest and most common one. This is typically done by extruding a suspension with probiotic bacteria in a hydrocolloid solution through a syringe needle into a gelling solution to obtain beads that have a diameter ranging from 2 to 5 mm (Krasaekoopt,

Bhandari, & Deeth, 2003). The dimension of beads is a drawback because calcium alginate beads bigger than 1 mm can cause sandy texture (Hansen, et al., 2002). The big dimension of beads also can limit the release rate of encapsulated bacteria (Krasaekoopt, et al., 2003). Smaller beads with a dimension from around 25 μm to 2 mm can be produced by the emulsion template method (Krasaekoopt, et al., 2003). This can be prepared by emulsifying a biopolymer (e.g., carrageenan, gelatin and locust bean gum) suspension with bacteria in oil to prepare water-in-oil (W/O) emulsions. The gelling agents are then mixed to form beads from water droplets. Although the emulsion template method is more advantageous than the extrusion method (Manojlović, Nedović, Kailasapathy, & Zuidam, 2010), these beads can also potentially cause the sandy texture because it can be detected when particulates bigger than about 10 μm are present in dairy products (Walstra, Walstra, Wouters, & Geurts, 2005).

The objective of the present work was to study solid/oil/water (S/O/W) emulsions as a novel approach to encapsulate *L. salivarius* NRRL B-30514 using spray-dried cells as the solid core, soybean oil as the oil phase, and SBP as a polymeric surfactant. This is the first time that SBP was applied to encapsulate probiotics in S/O/W emulsions with particles significantly smaller than millimeter-sized beads. *L. salivarius* is isolated from the digestive tract of mammals and has been used as a potential probiotic (Neville & O'Toole, 2010) with functions such as preventing the colonization of *Salmonella enteritidis* in chickens (Pascual, Hugas, Badiola, Monfort, & Garriga, 1999) and the infection by pathogenic *Listeria monocytogenes* (Corr, et al., 2007). The *L. salivarius* NRRL B-30514 in the present study is very effective in reducing *Campylobacter jejuni* in poultry (Stern, et al., 2006). In addition to physical properties of

emulsions, we studied the viability of encapsulated *L. salivarius* during storage, after thermal treatment, and after simulated gastrointestinal digestion.

## 4.3. Materials and methods

### *4.3.1. Materials*

SBP was a GENU® Explorer YA-400 product from CP Kelco (Atlanta, GA, USA). Soybean oil was purchased from MP Biomedicals, LLC. (Santa Ana, CA, USA). Pectinase from *Asperigillus niger* was purchased from MP Biomedicals, LLC. (Santa Ana, CA, USA). Other chemicals were from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

### *4.3.2. Emulsifying property of SBP*

#### *4.3.2.1. Preparation of O/W emulsions*

SBP was hydrated in distilled water on a stir plate overnight, and the obtained SBP solution had a pH of 3.9. After centrifugation at 5,372 *g* for 20 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT, USA) to remove impurities, the clarified SBP solution was used to prepare O/W emulsions by homogenization at 12,000 rpm for 4 min (IKA® 25 digital ULTRA TURRAX®, IKA® Works, Inc., Wilmington, NC, USA). Sodium azide (0.02% w/v) was added as an antimicrobial agent to prevent microbial spoilage. SBP concentrations in the aqueous phase were studied for 1.0%, 2.0%, 3.0% and 4.0% w/v, and the volume ratios of soybean oil and SBP

solution (O:W) were 1:4, 1:6, and 1:8. Emulsions were stored at 4 ℃ in a refrigerator if not analyzed immediately.

#### *4.3.2.2. Analysis of droplet dimension*

The dimension of emulsion droplets was measured using a LS 13 320 laser diffraction particle size analyzer (Beckman, Brea, CA, USA). Area-volume ($d_{3,2}$) and volume fraction-length ($d_{4,3}$) mean diameters were calculated using the following Eq. (4-1) and Eq. (4-2), respectively.

$$d_{3,2} = \frac{\sum_{i=1} n_i d_i^3}{\sum_{i=1} n_i d_i^2} \tag{4-1}$$

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \tag{4-2}$$

where $d_i$ is the diameter of the $i^{th}$ group of droplets and $n_i$ is the corresponding number of droplets.

#### *4.3.2.3. Evaluation of surface adsorption of SBP*

SBP adsorbed on the surface of emulsion droplets was measured after centrifuging emulsions at 15,000 *g* for 30 min using a MiniSpin Plus centrifuge (Eppendorf, Inc. Hauppauge, NY, USA). The serum phase was collected for determination of SBP concentration using a colorimetric method (Ye, Flanagan, & Singh, 2006). Briefly, 1 mL of the serum was mixed with 1 mL of a 5% w/w phenol solution and 5 mL of sulfuric acid (98%, w/w) and incubated for 10 min at room temperature (21 °C). After mixing by a vortex thoroughly, the mixture was

incubated for additional 30 min at room temperature before measuring absorbance at 485 nm with a UV/Vis spectrophotometer (model Evolution 201, Thermo Fisher Scientific, Waltham, MA, USA). Solution series with different SBP concentrations were used to construct a standard curve. Surface load ( $\Gamma_s$, mg/m$^2$ ) was then determined to evaluate the emulsifying property of SBP with the following Eq. (4-3) (Wu, Lin, & Zhong, 2014).

$$\Gamma_s = \frac{M_s \; d_{3,2}}{6V_{oil}} \tag{4-3}$$

where $M_s$ is the mass of SBP adsorbed on oil droplets, and $V_{oil}$ is the volume of soybean oil.

#### *4.3.2.4. Confocal laser scanning microscopy (CLSM)*

The structure of O/W emulsions was studied by CLSM. The microscope (model Leica TCS SP2, Leica Microsystems, Heidelberg GmbH, Germany) was equipped with three excitation sources, an Ar ion laser with 488 nm excitation wavelength and two HeNe lasers with excitation wavelengths of 543 and 633 nm. Before preparing O/W emulsions, SBP and soybean oil were labelled by fluorescent isothiocyanate (FITC) and Nile red, respectively. To label SBP, SBP was dissolved in sodium bicarbonate buffer at pH 9.0, and FITC was dissolved at a concentration of 0.4 mg/mL by stirring for 1 h at 21 °C (Twining, 1984). Soybean oil was mixed with a 0.1 mg/mL Nile red solution in propylene glycol at a volume ratio of 20:1 (Moschakis, Murray, & Dickinson, 2005). The labeled soybean oil and SBP solution were then used to prepare emulsions as above.

### *4.3.3. Encapsulation of L. salivarius*

#### *4.3.3.1. Preparation of spray-dried L. salivarius*

Stock *L. salivarius* culture from Department of Animal Science at the University of Tennessee (Knoxville, TN, USA) was inoculated in deMan, Rogoda and Sharpe (MRS) broth and incubated in an anaerobic jar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37 °C for 18 h. Cell pellets were collected by centrifugation at 5,372 *g* for 30 min (RC-5B Plus, Sorvall, Inc. Norwalk, CT, USA) at 4 °C. The pellets were washed with phosphate-buffered saline (PBS, pH 7.4) twice. The washed pellets were suspended at a population of about $10^8$ CFU/mL in skim milk additionally dissolved with 1% sucrose (Gardiner, et al., 2000). The suspension was spray dried using a B290 spray dryer (BÜCHI Corporation, Flawil, St. Gallen, Switzerland). The feed rate was 4.5 mL/min, the compressed air flow rate was about 400 L/h, the inlet temperature was 165 °C, and the outlet temperature was around 90 °C (Corcoran, Ross, Fitzgerald, & Stanton, 2004). The spray-drying operations resulted in a reduction of about 3 log/g dry matter. The spray-dried sample was denoted as free *L. salivarius* and stored at -20 °C before further experiments.

#### *4.3.3.2. Encapsulation of L. salivarius in S/O/W emulsions*

In encapsulation studies, the concentrations of SBP in aqueous solutions were studied for 3% and 4%, and the O:W volume ratios were studied at 1:6 and 1:8 for emulsions prepared with the 3% SBP solution and 1:4, 1:6, and 1:8 for the 4% SBP solution. Free *L. salivarius* was suspended in soybean oil at 1:20 w/v by mixing on a stir plate to obtain a visually homogenous

S/O suspension before homogenizing in the SBP solution as described in Section 2.2.1. In another set of emulsions after encapsulating *L. salivarius*, calcium chloride was dissolved at 0.1% w/v in the aqueous phase to cross-link SBP. Emulsions were stored at 4 °C before further experiments.

### *4.3.4. Enumeration of L. salivarius*

Free *L. salivarius* was serially diluted in 0.1% peptone solution, and then plated on MRS agar. Plates were incubated at 37 °C for 48 h in an anaerobic environment before enumeration.

For encapsulated *L. salivarius*, cells were released before enumeration. Preliminary experiments were used to monitor the hydrolysis of SBP by measuring reducing sugar content, and pectinase added at 0.5% w/v in emulsion adjusted to pH 4.0 was able to hydrolyze SBP completely in 15 min at 25 °C. After enzymatic hydrolysis, the released cells were collected by centrifugation at 15,280 *g* for 1 min (MiniSpin Plus centrifuge, Eppendorf, Inc. Hauppauge, NY, USA), and the resuspended cells were enumerated by plating as above. To release cells in calcium cross-linked emulsions, PBS at pH 8.0 was used to chelate calcium ions (Annan, Borza, & Hansen, 2008), followed by addition of pectinase and other procedures described previously.

### *4.3.5. Encapsulation efficiency (EE)*

The encapsulated *L. salivarius* was released and enumerated ($C_{emulsion}$) using the procedures in Section 4.3.4. The unencapsulated *L. salivarius* ($C_{free}$) was estimated by enumerating emulsions directly. The total live *L. salivarius* population used in encapsulation ($C_o$) was

determined by centrifuging the S/O suspension, resuspending the precipitated cells in the same volume of PBS (pH 7.4), and enumerating as in section 4.3.4. EE was then calculated (Eq. 4-4).

$$EE\% = \frac{Cemulsion(CFU/mL) - Cfree(CFU/mL)}{Co(CFU/mL)} \times 100\% \quad (4\text{-}4)$$

### *4.3.6. Storage stability of encapsulated L. salivarius*

The S/O/W emulsions with encapsulated *L. salivarius* were stored at 4 ℃. Free *L. salivarius* was used as a control by suspending in 0.1 M PBS (pH 3.9). The viable cell counts of all samples were enumerated by the method in Section 2.4 after storage at 4 °C for 0, 7, and 14 days.

### *4.3.7. Viability of encapsulated and free L. salivarius after pasteurization*

Samples were heated in a water bath, with the sample center being monitored for temperature using a thermometer. After the sample center reached 63 °C, samples were held for 30 min at 63 °C to simulate pasteurization (FDA, 2011). After cooling in a room-temperature water bath immediately, viable cell counts were determined using the methods in Section 4.3.4.

### *4.3.8. Survival of L. salivarius after in vitro digestions*

The S/O/W emulsions with encapsulated *L. salivarius* were evaluated for their survival after *in vitro* digestions. Free *L. salivarius* was used as a control by suspending in distilled water. The simulated gastrointestinal fluids were prepared according to the literautre with some modification (Chávarri, et al., 2010). The simulated gastric fluid was formulated with 3 g/L pepsin and 8.5 g/L sodium chloride, and the pH was 1.8. The simulated intestinal fluid was composed with 10 g/L pancreatin, 8.5 g/L sodium chloride, 3 g/L bile salt, and 10 g/L trypsin, and the pH was 6.5.

1 mL of an emulsion or control sample was mixed with 10 mL of the simulated gastric fluid and incubated at 37 ℃ for 2 h in a shaking water bath (New Brunswick Scientific Co., Edison, NJ, USA). During the incubation, 1 mL of the sample was withdrawn after 1 and 2 h incubation, the collected samples were adjusted to pH 4.0, and then viable cells in these samples were determined by the methods in Section 2.4. The sample after the simulated gastric digestion was dissolved with bile salt, trypsin, and pancreatin directly at aforementioned concentrations, and the pH was adjusted to 6.5. The mixture was incubated at 37 ℃ in the above shaking water bath for up to 4 h. During incubation, 1 mL of a sample was withdrawn at 1, 2, 3 and 4 h, and enumerated for survival cell counts as described above.

#### *4.3.9. Statistical analysis*

Mean and standard deviation were calculated from triplicates. One-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL).

## 4.4. Results and discussions

### *4.4.1. Emulsifying property of SBP*

The emulsifying property was evaluated by measuring droplet size ($d_{3,2}$) of emulsions and determining surface load of SBP ( $\Gamma_s$). The $d_{3,2}$ of emulsions is shown is Figure 4-1. With the same O:W volume ratio, $d_{3,2}$ decreased significantly with the increase of SBP concentration ($P < 0.05$), except for no difference between emulsions prepared with 3% and 4% SBP at the O:W volume ratio of 1:8. This is expected because a sufficient amount of SBP is needed to adsorb on

emulsion droplets during emulsification to prevent coalesce (Abismaïl, Canselier, Wilhelm, Delmas, & Gourdon, 1999). Because emulsions with smaller droplets have a better stability against gravitation separation (McClements, 1998), encapsulation of *L. salivarius* was studied at O:W volume ratios of 1:6 and 1:8 for 3% SBP, and 1:4, 1:6, and 1:8 for 4% SBP.

The $\Gamma_s$ of SBP was higher at a higher SBP concentration at the same O:W volume ratio (Figure 4-1). The observation generally agreed with an earlier study using different concentrations of SBP to emulsify 20% v/v rapeseed oil or orange oil (Leroux, et al., 2003). The $\Gamma_s$ values in the present study were much higher (up to 40 mg/m$^2$ when the emulsion was prepared with 4% SBP at the O:W volume ratio of 1:8) than the 1-3 mg/m$^2$ expected for protein monolayers (Dickinson, 1997). A $\Gamma_s$ value of 9.16 mg/m$^2$ was reported for soybean oil emulsified by preheated soy protein that formed protein aggregates (Cui, Chen, Kong, Zhang, & Hua, 2014). The relatively high $\Gamma_s$ in the present study possibly is because SBP has a small fraction of protein and the glycated polysaccharide chains increase the molar mass of polymeric surfactants adsorbing on oil droplets (Leroux, et al., 2003).

Figure 4-2 shows the CLSM micrographs of emulsions prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6. The oil droplets were covered by SBP. Most droplets were smaller than 10 μm, which agreed with the light scattering results in Figure 4-1.

### *4.4.2. Efficiency of encapsulating L. salivarius in S/O/W emulsions*

EEs of treatments are shown in Table 4-1. Overall, the highest EE of 87% was observed for the treatment with 3% w/v SBP and an O:W volume ratio of 1:8. When the SBP concentration

was 3%, the EE was higher at the O:W volume ratio of 1:8 than the treatment with a volume ratio of 1:6 but the difference was not significant ($P$>0.05). Similarly, the emulsions prepared with 4% SBP had a higher EE at a lower O:W volume ratio, and the treatment with a volume ratio of 1:8 had a significantly higher EE ($P$ < 0.05) than the other two volume ratios and was not different ($P$ > 0.05) from the treatments prepared with 3% SBP. The higher EEs in Table 4-1 generally agreed with the higher $\Gamma_s$, which highlights the significance of SBP adsorption during emulsification on encapsulating bacteria in oil droplets. The rest of experiments were conducted for emulsions prepared with 3% SBP and an O:W volume ratio of 1:6.

### *4.4.3. Viability of L. salivarius encapsulated in S/O/W emulsions during storage*

The viability of *L. salivarius* during storage at 4 ℃ is presented in Table 4-2. The reduction of *L. salivarius* was significant ($P$ < 0.05) for all treatments after two-week storage. The reduction was about 5.4 log CFU/mL for the free cell suspension, 2.2 log CFU/mL for the emulsion, and only 1.4 log CFU/mL for the emulsion with $CaCl_2$. Various factors can attribute to the survival of probiotics during storage, such as temperature, oxygen content, and contents of lactic and acetic acids (Dave & Shah, 1997; Kailasapathy & Rybka, 1997). Encapsulation of probiotics can reduce the impacts of adverse environmental stresses and potentially reduce the cell inactivation. Encapsulation of *Bifidobacterium pseudolongum* in cellulose acetate phthalate, a biopolymer insoluble at gastric acidity, improved the survival of probiotics in simulated gastric conditions (Rao, Shiwnarain, & Maharaj, 1989). In another study, encapsulation of *L. acidophilus* and *Bifidobacterium* spp. in calcium alginate beads enhanced the survival of these

probiotics in yogurt during 8-week storage at 5 ℃, with the reduction improving for about 0.5 log CFU/mL when compared to the unencapsulated probiotics (Sultana, et al., 2000). In our study, spray-dried *L. salivarius* particles were encapsulated in oil droplets that are separated from the aqueous phase, which can similarly improve the viability of bacteria. Cross-linking surface SBP by divalent calcium ions strengthens the interfacial layer structure that improves the stability of oil droplets and the viability of encapsulated bacteria during storage.

### *4.4.4. Viability of L. salivarius encapsulated in S/O/W emulsion after pasteurization*

The viable cell counts of free and encapsulated *L. salivarius* after heating at 63 °C for 30 min are summarized in Table 4-3. After pasteurization, the viable cell count of free *L. salivarius* suspension was below the detection limit, which agrees with the poor thermal tolerance of *L. salivarius* (Gardiner, et al., 2000). The reduction of *L. salivarius* encapsulated in S/O/W emulsion with and without $CaCl_2$ was about 5.4, and 4.7 log CFU/mL, respectively, and was not significantly different ($P > 0.05$). As presented previously, encapsulation of *L. salivarius* in oil droplets can reduce the thermal stress and improve the survival after pasteruzation, because the exclusion of water molecules around bacteria is one mechanism reducing the death of bacteria during heating (Daemen, 1981).

### *4.4.5. Droplet dimension of S/O/W emulsions with encapsulated L. salivarius*

Table 4-4 shows the $d_{4,3}$ of S/O/W emulsions with encapsulated *L. salivarius* with and without calcium cross-linking, before and after storage and pasteurization. The $d_{4,3}$ of fresh emulsion increased from 7.32 μm to 16.73 μm after addition of $CaCl_2$, indicating the possible

ionic bridging of emulsion droplets and also possibly free SBP. The $d_{4,3}$ significantly decreased ($P < 0.05$) after storage and after pasteurization, which resulted from destabilization of big emulsions droplets as evidenced by free oil for some samples. The reduction of droplet dimension was less significant for the emulsion without $CaCl_2$ (<2 μm) than that with $CaCl_2$, especially after heating (a reduction of ~6 μm). This can be caused by the separation of flocculated droplets initially cross-linked by calcium ions. The storage and thermal stability of emulsions can be further studied for other $CaCl_2$ concentrations or layer-by-layer deposition of polyelectrolytes, which were not attempted in the present study. Overall, the dimension of S/O/W emulsion droplets being smaller than 17 μm is an important feature of applying encapsulated probiotics in dairy products such as yogurt, as discussed previously.

### *4.4.6. Viability of L. salivarius during in vitro digestion*

The viability of free and encapsulated *L. salivarius* was studied in the simulated gastric fluid for 2 h followed by incubation in the simulated intestinal fluid for 4 h. The viable cell counts of treatments are presented in Figure 4-3. During the first 2 h incubation in the simulated gastric fluid, all treatments experienced a gradual reduction of viable cell counts. The viable cell count decreased from 6.86 to 4.46 log CFU/mL for free *L. salivarius*, from 6.70 to 4.78 log CFU/mL for the emulsion without $CaCl_2$, and from 6.62 to 5.92 log CFU/mL for the emulsion with $CaCl_2$. Although *Lactobacillus* species are considered to be intrinsically acid-resistant and *L. salivarius* have a relatively high acid tolerance (Ding & Shah, 2009), the tolerance of *L. salivarius* is limited at gastric conditions (Charteris, Kelly, Morelli, & Collins, 1998).

Consequently, the significant reduction was observed for free *L. salivarius* in the simulated gastric fluid. The exclusion from stresses in the gastric fluid improved the viability of *L. salivarius* after encapsulation in oil droplets, especially after cross-linking by calcium. The observations in Figure 3 agree with a study about *L. gasseri* and *Bifidobacterium bifidum* encapsulated in alginate microspheres surface-coated with cationic chitosan (Chávarri, et al., 2010). The inactivation of *L. salivarius* can be attribute to demulsification after the hydrolysis of proteinaceous moiety of SBP by pepsin (Funami, et al., 2007). Additionally, it can also result from the portion of unencapsulated bacteria. Protonation may also change the conformation of SBP at gastric acidity to impact the emulsion droplet stability, which is alleviated after calcium cross-linking.

When samples were further incubated in the simulated intestinal fluid, the viable cell counts of free *L. salivarius* became undetectable in 1 h. The population of viable *L. salivarius* encapsulated in the S/O/W emulsion decreased from 4.70 to 1.69 log CFU/mL after 2 h incubation and became undetectable afterwards. For the S/O/W emulsion treatment with $CaCl_2$, the gradual reduction of viable *L. salivarius* from 5.92 log CFU/mL was also observed, but 2.65 log CFU/mL of viable cells was observed after 4-h incubation. The results in Figure 3 can be attributed by proteolysis of the protein fraction of SBP by proteases (NUNez, Fishman, Fortis, Cooke, & Hotchkiss Jr, 2009) and the subsequent demulsification to release the encapsulated *L. salivarius* that is then quickly inactivated by bile salt (Binder, Filburn, & Floch, 1975). Cross-linking surface SBP by calcium ions strengthens the interfacial films and reduces the digestion by proteases (Smidsrød, 1990). Because pectin can be degraded by the native microflora in the

colon (Liu, Fishman, Kost, & Hicks, 2003), the residual bacteria can be released after passing through the small intestine.

## 4.5. Conclusions

In conclusion, SBP can be used to prepare S/O/W emulsions to encapsulate *L. salivarius* with an EE of up to about 87% and droplets smaller than 17 μm. Encapsulation of *L. salivarius* in S/O/W emulsions improved the viability during storage, pasteurization, and *in vitro* digestions. Cross-linking SBP on emulsion droplets by divalent calcium ions additionally improved the viability of *L. salivarius* against various environmental stresses deactivating the bacterium, enabling the survival of a significant portion of viable *L. salivarius* after simulated gastrointestinal digestions. Future *in vivo* studies are needed to verify the studied S/O/W emulsions as a potential system to deliver probiotic bacteria in foods to improve their viability during processing, storage, and digestion.

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## Appendix

Table 4-1. Efficiency of S/O/W emulsions encapsulating *L. salivarius* at different O:W volume ratios and SBP concentrations.

| SBP concentration in the aqueous phase (% w/v) | O:W volume ratio | Encapsulation efficiency (%)* |
|---|---|---|
| 3 | 1:6 | 67.96±9.61[a] |
| | 1:8 | 87.37±12.36[a] |
| 4 | 1:4 | 29.13±6.87[b] |
| | 1:6 | 37.38±14.42[b] |
| | 1:8 | 67.72±3.09[a] |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 4-2. Viability of free and encapsulated *L. salivarius* during storage at 4 ℃.

| Storage time (day) | Viable cell count (log CFU/mL)* | | |
|---|---|---|---|
| | Free *L. salivarius* | S/O/W emulsion[#] | S/O/W emulsion with calcium[#] |
| 0 | 7.66±0.19[a] | 6.89±0.21[b] | 6.35±0.04[c] |
| 7 | 3.95±0.0.18[g] | 5.61±0.03[d] | 5.73±0.07[d] |
| 14 | 2.30±0.33[h] | 4.71±0.09[f] | 4.95±0.07[e] |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

[#] The emulsion was prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6, with and without 0.1% $CaCl_2$.

Table 4-3. Viability of free and encapsulated *L.salivarius* after pasteurization at 63 ℃ for 30 min.

| Samples | Viable cell count (log CFU/mL)* | |
|---|---|---|
| | Before heating | After heating |
| Free *L.salivarius* suspension | 7.66±0.19[a] | <1 |
| Emulsion[#] | 6.89±0.21[b] | 1.48±0.14[d] |
| Emulsion with calcium[#] | 6.36±0.04[c] | 1.78±0.02[d] |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$). The detection limit is 1 log CFU/mL.

[#] The emulsion was prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6, with and without 0.1% $CaCl_2$.

Table 4-4. Volume fraction-length mean diameter ($d_{4,3}$) of S/O/W emulsions with encapsulated *L. salivarius* before and after storage at 4 ℃ and pasteurization at 65 ℃ for 30 min.

| Treatment | $d_{4,3}$ (μm)* | |
|---|---|---|
| | Emulsion [#] | Emulsion with calcium[#] |
| Fresh | 7.32±0.03[e] | 16.73±0.10[a] |
| 7-day storage | 5.52±0.01[g] | 13.44±0.08[b] |
| 14-day storage | 5.33±0.04[h] | 12.14±0.03[c] |
| Pasteurization | 6.15±0.01[f] | 10.41±0.08[d] |

*Numbers are mean ± standard deviation from triple measurements. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

[#] The emulsion was prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6, with and without 0.1% $CaCl_2$.

Figure 4-1. Effects of SBP concentration in the aqueous phase on the volume-area mean diameter (d3,2, circles) and surface load of SBP ($\Gamma$s, squares) for emulsions prepared with O:W volume ratios of (A) 1:4, (B) 1:6, (C) 1:8. Different letters next to the symbols indicate significant difference in the mean of surface load (uppercase) or mean diameter (lower case) for all samples ($P < 0.05$).

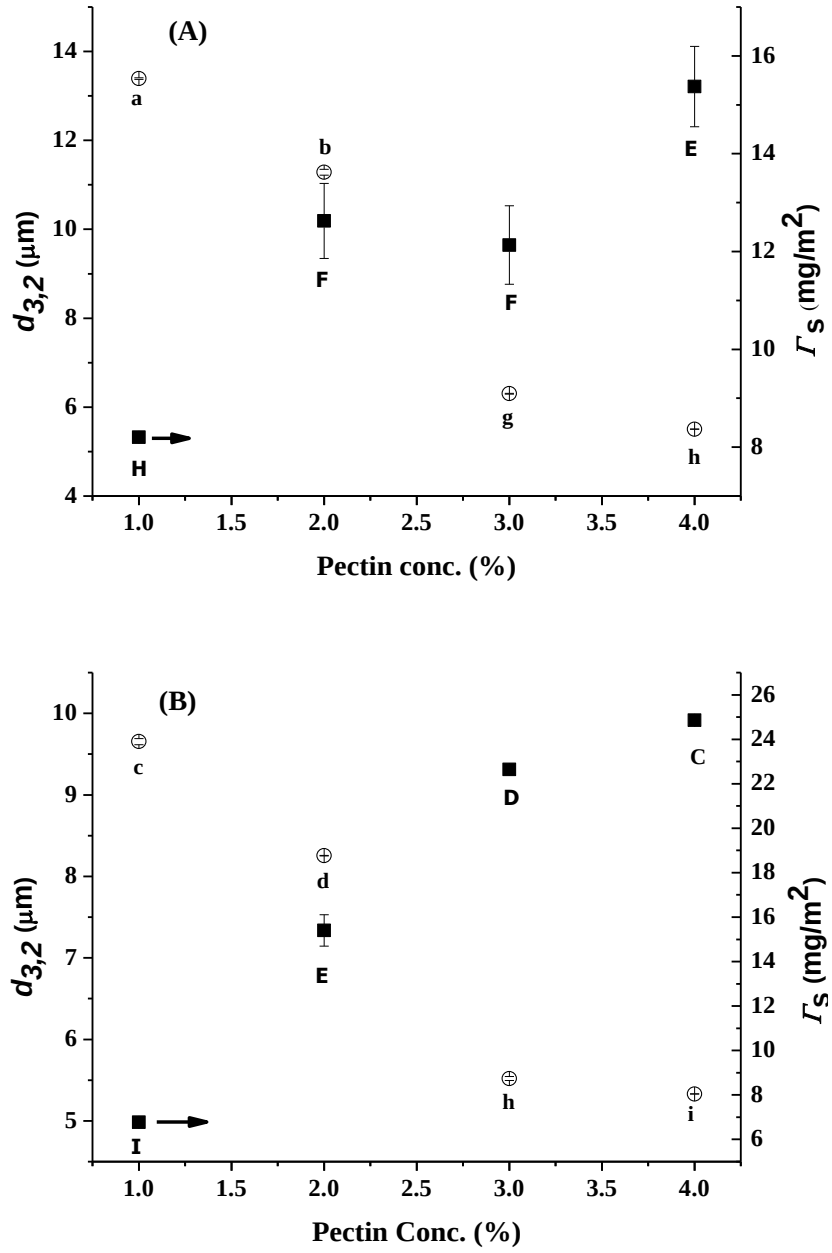


Figure 4-1. continued

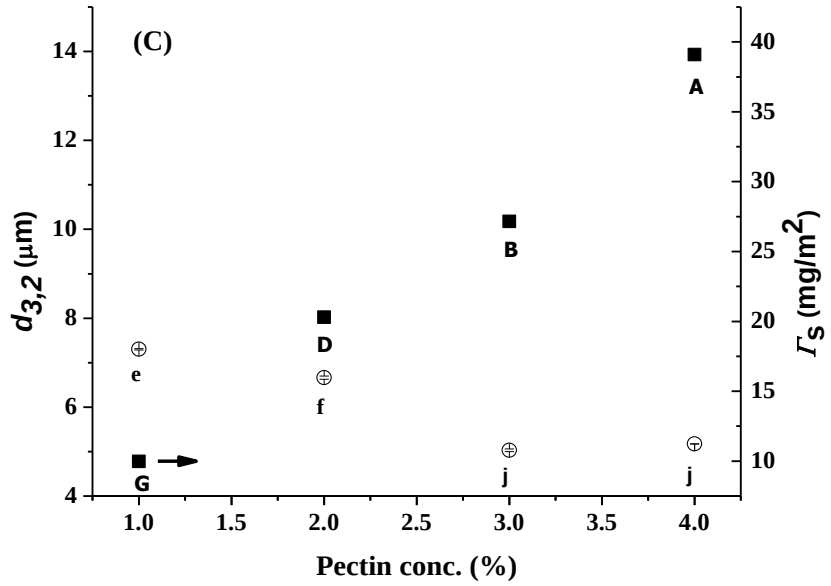


Figure 4-1. continued

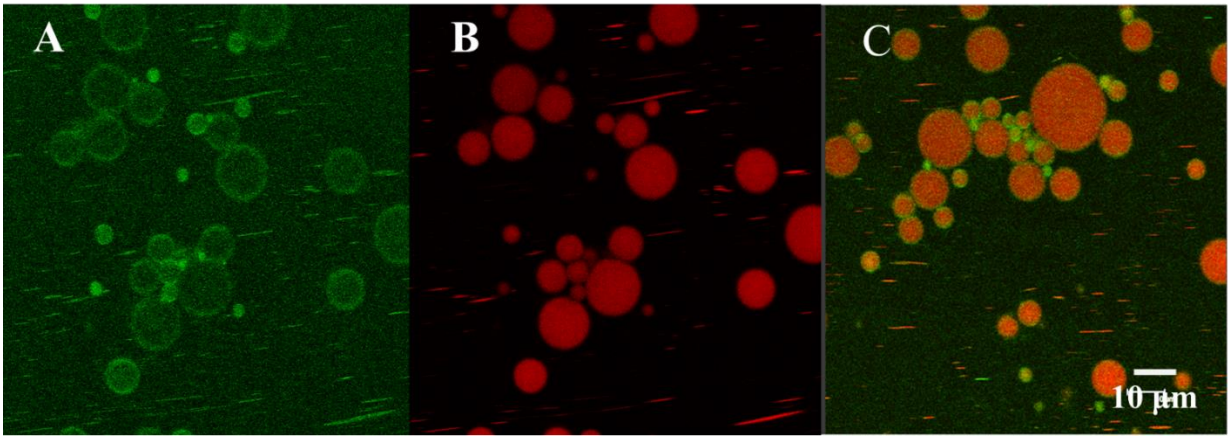


Figure 4-2. Confocal laser scanning micrographs of O/W emulsion prepared with SBP: (A) green fluorescence showing FITC-labeled SBP; (B) red fluorescence showing Nile red labeling oil; (C) a merged micrograph showing both. The emulsion was prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6.

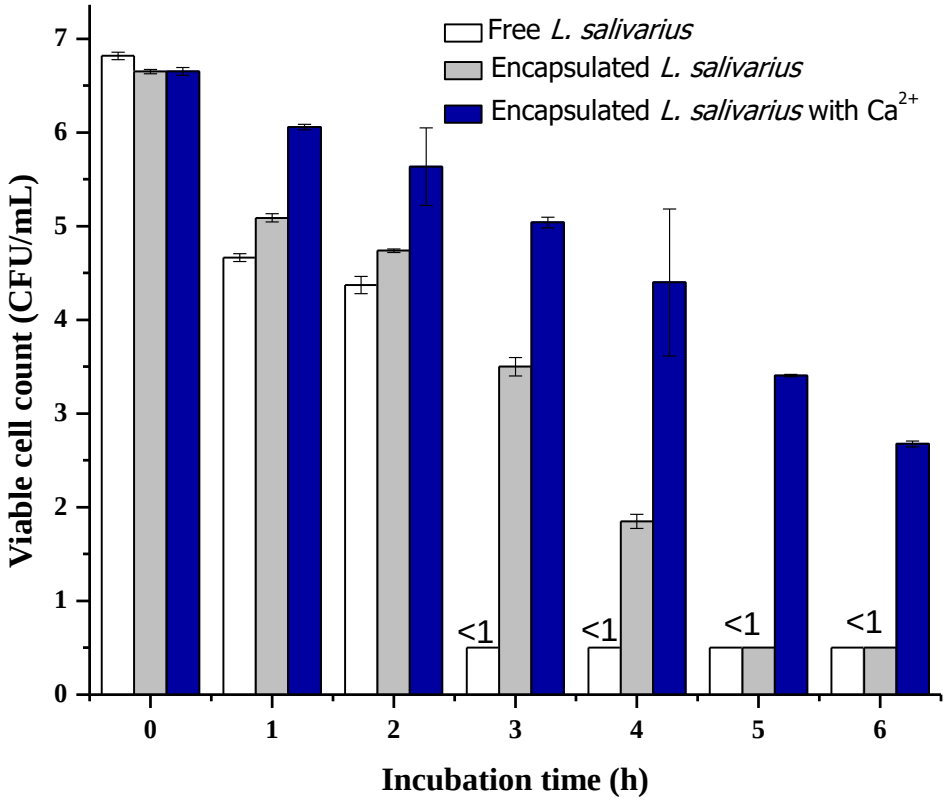


Figure 4-3. Viable cell counts of free and encapsulated L. salivarius during simulated gastric (first 2 h) and intestinal (3-6 h) digestions. Data labelled "<1" denote the viable cell counts are below the detection limit of 1 log CFU/mL. The emulsion was prepared with 3% SBP in the aqueous phase and an O:W volume ratio of 1:6, with and without 0.1% $CaCl_2$. Error bars are standard deviations from triple measurements.

# Chapter 5 . Effects of media, heat adaptation, and outlet temperature on the survival of *Lactobacillus salivarius* NRRL B-30514 after spray drying and subsequent storage

## 5.1. Abstract

Production of spray-dried powder with viable probiotic bacteria is affected by many factors. The objective of this work was to study the viability of probiotic *Lactobacillus salivarius* NRRL B-30514 strain, with or without prior heat adaptation, after spray drying with different media and outlet temperatures and during subsequent storage in a desiccator at 21°C. The media included 20%w/v reconstituted skim milk (RSM), 18%w/v RSM+4%w/v sucrose+4%w/v trehalose (RSMST), and 18%w/v RSM+4%w/v lactose+4%w/v trehalose (RSMLT). The outlet temperatures were studied for 98-100, 94-96, 90-92, 84-86, 80-82, 74-76, and 70-72 ℃. The heat adaptation was studied by heating the cell suspensions at 50 ℃ for 15 min before spray drying. Overall, the reduction of viable *L. salivarius* in all media with and without heat adaptation decreased with the decrease of outlet temperature, and the highest outlet temperature (98-100 ℃) caused 4.54 log CFU/g reduction for the RSM treatment without heat adaptation. After spray drying at the lowest outlet temperature (70-72 ℃), the lowest (0.56 log CFU/g) and highest (2.16 log CFU/g) reduction was observed for cells suspended in RSMLT with heat adaptation and those in RSM without heat adaptation, respectively. During storage, the reduction of viable *L. salivarius* was lower for the powder spray-dried with a higher outlet temperature, and the powder prepared with RSM had the lowest survivability. The storage stability was correlated to the lower water activity of spray-dried powder (0.109-0.246) produced at a higher outlet temperature. Therefore, the studied parameters can be combined to improve the production of spray-dried probiotics.

**Keywords**: spray-dried probiotics, viability, heat adaption, media, outlet temperature, storage

## 5.2. Introduction

Probiotic bacteria are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hotel & Cordoba, 2001). To date, probiotic bacteria have been verified to have a positive influence on alleviation of lactose intolerance, stimulation of gastrointestinal tract immune system, resistance to intestinal infection, suppression of cancer, interference of cholesterol adsorption, assistance to digestion, among others (Fooks, Fuller, & Gibson, 1999). Fermented dairy products are the most common carrier of probiotics. However, fermented dairy products have a short shelf life and require refrigerated storage and transportation that increase costs of incorporating probiotics. These limitations may be overcome by dehydrating probiotics.

The most common dehydration methods are freeze-drying and spray-drying (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). Freeze-drying is favored to maintain the viability of probiotics but is costly and time-consuming (Gardiner, O'sullivan, Kelly, Auty, Fitzgerald, Collins, et al., 2000). In contrast, spray-drying is a low cost and high yield dehydration technology, but inactivates probiotics (Chávez & Ledeboer, 2007). The mortality of probiotic bacteria during and after spray-drying is attributed to various factors, such as media, stress treatment, drying temperature, and water activity that impact not only the extent of thermal inactivation but also the loss of water bound on the cell surface (Daemen & Van der Stege, 1982). Therefore, optimization of drying parameters, selection of drying medium, and application of environmental adaptation is needed for each probiotic bacterium. However, most studies focus on optimizing

one single parameter, and there is a need to improve the viability of probiotics with combinations of multiple strategies.

The first objective of the present study was to investigate the viability of probiotic *L. salivarius* NRRL B-30514, with or without prior heat adaptation, after spray drying with different media and outlet temperatures. The second objective was to study the viability of spray-dried bacteria during storage in a desiccator at 21 °C. *L. salivarius* NRRL B-30514 was isolated from chicken intestine and has the function of reducing *Campylobacter jejuni* in poultry (Stern, Svetoch, Eruslanov, Perelygin, Mitsevich, Mitsevich, et al., 2006; Wang, Zeng, Mo, Smith, Guo, & Lin, 2012). This is the first study about the viability of spray-dried *L. salivarius* NRRL B-30514, and the optimization of parameters will directly facilitate the production of feedstuff and therefore animal foods. Reducing the outlet temperature is expected to improve the viability of probiotics during spray drying (Gardiner, et al., 2000). Skim milk powder, sucrose, lactose, and trehalose were studied as drying media alone or in combination, as these compounds are commonly added in media to protect the viability of probiotic bacteria during drying (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). Sub-lethal heat treatment (adaptation) can improve the thermal tolerance of bacteria (Colette Desmond, Stanton, Fitzgerald, Collins, & Paul Ross, 2001; Teixeira, Castro, & Kirby, 1994). By controlling water activity of spray-dried powder to be between 0.11 and 0.23, the cell death of spray-dried probiotic bacteria during storage can be reduced (Chávez & Ledeboer, 2007).

## 5.3. Materials and methods

### *5.3.1. Materials*

Organic nonfat dry milk powder was from Organic Valley Family of Farms (La Farge, WI, USA). D-trehalose dihydrate (99%) was from Cascade Analytical Reagents and Biochemicals (Corvallis, OR, USA). Sucrose and D-lactose monohydrate were purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

### *5.3.2. Bacterial strain and culture conditions*

The stock culture of *L. salivarius* NRRL B-30514 was from Department of Animal Science at the University of Tennessee (Knoxville, TN, USA). The 1 mL frozen stock culture in glycerol was subcultured in 280 mL deMan, Rogoda and Sharpe (MRS) broth at 37 °C overnight (~18 h) under anaerobic conditions that were obtained in an anaerobic jar with an anaerobe container system sachet (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells were collected by centrifugation at 3000 *g* for 30 min using a RC-5B Plus centrifuge (Sorvall, Inc. Norwalk, CT, USA) at 4 °C, and the harvested cells were washed twice with a phosphate-buffered saline (PBS, at pH 7.4). The washed cells were suspended in a drying medium prepared as below.

### *5.3.3. Preparation of drying media*

Reconstituted skim milk (RSM) was prepared with 20% w/v of non-fat dry milk in deionized water, as a control medium. RSM with additional sucrose and trehalose (RSMST) was prepared with 18% w/v non-fat dry milk, 4% w/v sucrose and 4% w/v trehalose. RSM with lactose and trehalose (RSMLT) was prepared similarly to RSMST, by substituting sucrose for

the same concentration of lactose. All media were sterilized in a water bath at 90 °C for 30 min (Ananta, Volkert, & Knorr, 2005) before use.

### *5.3.4. Determination of sub-lethal temperatures of L. salivarius NRRL B-30514*

Sub-lethal temperature was measured using a literature method (Anekella & Orsat, 2013) with modification. Briefly, the exponential phase culture in various drying media (RSM, RSMST and RSMLT) was collected, 5 mL of the culture was heated at 45, 50, 52 and 55 °C, and 1 mL aliquots were withdrawn at time intervals of 0, 5, 10, and 15 min. After cooling in a room-temperature water bath immediately, viable cell counts were determined with the pour plating method on MRS agar.

### *5.3.5. Preparation of spray-dried L. salivarius NRRL B-30514*

Washed cells prepared as in Section 2.2 were suspended at about $3 \times 10^8$ CFU/mL in a drying medium as described in Section 2.3. All cell suspensions were spray-dried using a laboratory scale spray dryer (model B290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) at a constant inlet temperature of 170 °C. The effect of outlet temperature on the viability of spray-dried *L. salivarius* was studied by changing the pump rate to obtain outlet temperatures of 98-100, 94-96, 90-92, 84 -86, 80-82, 74-76, and 70-72 °C.

Viable cell counts in spray-dried powder were determined using the pour plating method. A spray-dried sample was reconstituted at 1 mg/mL in the maximum recovery diluent (peptone saline broth) (Thermo Scientific™ Oxoid™, Remel Inc, Lenexa, KS, USA) for 1 h at room temperature. The serially diluted samples were then pour plated on MRS agar. The plates were

incubated at 37 °C for 48 h in anaerobic conditions before enumeration. Two spray-dried samples were determined in duplicate (n = 4).

To investigate the effect of heat adaptation, washed *L. salivarius* cultured overnight anaerobically at 37 ºC was inoculated at 1% v/v in a drying medium with a cell density of ~$10^6$ CFU/mL and incubated at anaerobic conditions at 37 °C for about 5 h to obtain an exponential phase culture, followed by incubation in a water bath at 50 °C (determined as the sub-lethal temperature in the present study) for 15 min (Colette Desmond, Stanton, Fitzgerald, Collins, & Paul Ross, 2001; W. S. Kim, Perl, Park, Tandianus, & Dunn, 2001). Cultures after heat adaptation were spray-dried with the same parameters mentioned previously. Viable cell counts of spray-dried powder were enumerated using the above pour plating method.

### *5.3.6. Scanning electron microscopy of spray-dried powder*

Spray-dried powder with *L. salivarius* was glued onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of about 5 nm in thickness. Samples were imaged using a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany).

### *5.3.7. Water activity of spray dried powder*

The water activity of spray dried powders was determined with an AquaLab Series 3TE instrument (Decagon Devices, Inc., Pullman, WA, USA).

### *5.3.8. Viability of spray-dried L. salivarius during storage*

Spray-dried *L. salivarius* was collected to vials and stored in a desiccator at room temperature (21 ºC). Viable cells in the powder after storage were enumerated with the method presented in Section 5.3.5.

### *5.3.9. Statistical analysis*

Means and standard deviations were calculated from two independent spray-dried sample replicates. A one-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL). The least-significant-difference (LSD) test was used to determine the significant differences of mean at a *P* level of 0.05.

## 5.4. Results and discussions

### *5.4.1. Sub-lethal temperature of L. salivarius NRRL B-30514*

Viable cell counts after incubation in three different drying media at 45, 50, 52 and 55 °C are shown in Figure 5-1. For all drying media, viable cell counts of *L. salivarius* were decreased dramatically after heating at 52 and 55 °C for 5 min. However, when samples were heated at 45 or 50 °C for up to 15 min, no significant ($P$ <0.05) changes of viable cell counts were observed. Consequently, 50 °C is considered as the sub-lethal temperature of *L. salivarius* NRRL B-30514 (W. S. Kim, Perl, Park, Tandianus, & Dunn, 2001). In the following experiments, heating at 50 °C for 15 min was used for heat adaptation treatments.

### *5.4.2. Effects of drying media on the survivability of L. salivarius NRRL B-30514 after spray drying*

Reductions of *L. salivarius* suspended in RSM, RSMST, or RSMLT after spray drying at various outlet temperatures are shown in Table 5-1. The reductions of *L. salivarius* spray-dried in RSMLT were significantly lower ($P<0.05$) than those in RSM and RSMST, while treatments in RSM and RSMST were not significantly different ($P \geq 0.05$) with an exception at an outlet temperature of 90-92 °C that resulted in a significantly higher ($P < 0.05$) reduction of viable *L. salivarius* in RSMST than in RSM.

It has been reported that sugars such as sucrose and trehalose can enhance the viability of bacteria during dehydration by stabilizing membranes and proteins (Rudolph & Crowe, 1985), partially because disaccharides form hydrogen bonds to maintain the tertiary structures of proteins during drying (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995). It was also proposed that physical properties of drying media such as thermal conductivity and diffusivity can influence bacteria survivability (Lian, Hsiao, & Chou, 2002). The positive impacts of disaccharides on the viability of *L. salivarius* after spray drying however were observed for RSMLT not RSMST in the present study. The difference between treatments with lactose and sucrose may be attributed to the much higher glass transition temperature ($T_g$) of α-lactose monohydrate (101 °C) than sucrose (66 °C) (Christensen, Pedersen, & Kristensen, 2002; Roos, 1993). During spray drying, stickiness is likely if the outlet air temperature is 10 °C higher than

the $T_g$ of disaccharides (Vega & Roos, 2006). It could be that the higher $T_g$ of RSMLT than RSMST reduce the stickiness during spray drying to improve the survivability of bacteria.

### *5.4.3. Effects of outlet temperature on the survivability of L. salivarius NRRL B-30514 after spray drying*

When treatments prepared at different outlet temperatures were compared, viable cell counts increased significantly ($P < 0.05$) when the outlet temperature decreased from 98-100 to 94-96 °C for all drying media (Table 5-1). However, when the outlet temperature decreased from 94-96 to 90-92 °C, the reductions of viable cell counts did not change significantly ($P \geq 0.05$). Compared to treatments at an outlet temperature of 90-92 °C, spray drying *L. salivarius* in RSM and RSMLT at an outlet temperature of 84-86 °C alleviated the reduction of viable cell counts significantly ($P < 0.05$), while it was not significant for the treatment in RSMST ($P \geq 0.05$). Further reductions of the outlet temperature from 84-86 to 80-82 °C and from 80-82 to 74-76 °C resulted in significant increases of viable cells in RSMST and RSMLT treatments ($P<0.05$), but not in RSM treatments ($P \geq 0.05$). Finally, the reduction of viable cells did not decrease significantly when lowering the outlet temperature further from 74-76 to 70-72 °C ($P \geq 0.05$). Overall, the reduction of viable cells was less significant at a lower outlet temperature when spray drying *L. salivarius* in the same medium.

During spray drying, reduction of viable cells is mainly attributed to the lethal thermal injury (To & Etzel, 1997). Various studies concluded that an increase of outlet temperature reduces the survivability of bacteria during spray drying. For example, the viability of *L. acidophilus* had an

obvious decrease when spray drying in reconstituted nonfat dry milk at an increased outlet temperature (Espina & Packard, 1979). The percentage of viable *Brevibacterium linens* spray-dried in condensed skim milk decreased by about 50% with every 5 °C increase of outlet temperature (To & Etzel, 1997). During heating, not only are membranes and ribosomes damaged, but also DNA, RNA and proteins (Abee & Wouters, 1999), and a severe damage of a critical component is sufficient to inactivate bacteria (C Santivarangkna, Kulozik, & Foerst, 2008).

### *5.4.4. Effects of heat adaptation on the survivability of L. salivarius after spray drying*

The reductions of viable cells after drying at various outlet temperatures in three media with and without heat adaptation are shown in Table 5-1. Overall, with the same drying medium, the reductions of viable cells spray-dried in RSMST and RSMLT significantly decreased ($P < 0.05$) after heat adaptation at all outlet temperatures, and the lowest reduction was 0.56 log CFU/g for *L. salivarius* spray dried in RSMLT with heat adaptation at an outlet temperature 70-72 °C. In RSM, such a significant improvement in the viability of *L. salivarius* after heat adaption was only observed at outlet temperatures of 98-100 and 94-96 °C ($P < 0.05$). These results indicate that heat adaptation can improve the viability of *L. salivarius* during spray drying, but the extent of improvement is a function of drying media and spray drying conditions. Similar observations have been reported by others (Colette Desmond, Stanton, Fitzgerald, Collins, & Paul Ross, 2001; Teixeira, Castro, & Kirby, 1994).

Various mechanisms are proposed to explain the effects of heat adaptation on the viability of bacteria. One theory hypothesized that the increased saturation and length of fatty acids after heat adaptation can maintain the activity of intrinsic proteins and the fluidity of the membrane (Russell & Fukunaga, 1990). Another theory proposes the production of heat shock proteins being responsible for the positive effect of heat adaptation because heat shock proteins are related to the precise folding of nascent polypeptides, assembly of protein complexes, degradation and translocation of proteins (Bukau & Horwich, 1998; De Angelis & Gobbetti, 2004). The experimental observation about the effects of heat adaptation on cell survivability is affected by factors such as growth media of bacteria, culture age, and shock media, and heat adaptation in a complex medium can improve the thermal tolerance of bacteria than in a buffer (Teixeira, Castro, & Kirby, 1994). Although the exact mechanisms were not studied in the present study, results in Table 5-1 generally agreed with the above studies.

### *5.4.5. Morphology of spray-dried powder*

Scanning electron micrographs of *L. salivarius* spray-dried in the three media are shown in Figure 5-2. Some particles were spherical, while most particles appeared to have the collapsed structures. The dimension of particles was quite heterogeneous and appeared to follow the decreasing order of RSM, RSMLT, and RSMST. Addition of disaccharides to the drying media increases the solids content that is expected to increase the size of spray-dried powder particles (Paramita, Iida, Yoshii, & Furuta, 2010). In addition, the drying front of atomized droplets with materials of a lower $T_g$ can continue for a longer time to evaporate water before the formation of

crust. Skim milk powder has a $T_g$ close to that of lactose (Jouppila & Roos, 1994), while D-(+)-trehalose dehydrate has a higher $T_g$ of 114.9 °C (Miller, de Pablo, & Corti, 1997). Therefore, $T_g$ of drying media follows the increasing order of RSMST < RSM < RSMLT. The much lower $T_g$ of sucrose (66 °C) than other drying medium components (above 100 °C) (Christensen, Pedersen, & Kristensen, 2002; Roos, 1993) may have been responsible for the smallest particles of RSMST, while the higher solids content of RSMLT than RSM may have resulted in their difference in particle dimension. A few particles produced with RSM also showed cracks, similar to a previous report of *B. longum* B6 spray-dried in skim milk (Lian, Hsiao, & Chou, 2002). Addition of disaccharides in the drying media increased the solids content, corresponding to particles with smooth surfaces (Paramita, Iida, Yoshii, & Furuta, 2010) due to the increased viscosity slowing down the redistribution of components and the accelerated crust or skin formation during drying of individual droplets (E. H. J. Kim, Chen, & Pearce, 2009).

### *5.4.6. Water activity of spray-dried powder*

The water activity of all spray-dried samples is summarized in Table 5-2. Overall, water activity was higher for the sample produced at a lower outlet temperature, with the exception for the RSMLT treatments without heat adaptation at outlet temperatures of 74-76 and 70-72 °C. Besides, the water activity of treatments with heat adaptation was lower than the comparable treatments with the same medium and outlet temperature. The results in Table 5-2 are in agreement with the reported lower water activity of bacteria spray-dried at a higher outlet

temperature (C. Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002; Colette Desmond, Stanton, Fitzgerald, Collins, & Paul Ross, 2001).

Dehydration is a major mechanism causing the mortality of bacteria during spray drying (Janning & In't Veld, 1994). Water molecules are critical to the stability of proteins, lipids and DNA of bacteria, and the reduction of water content impacts physiological functions of bacteria, eventually influencing various cellular components when the water content is sufficiently low (Peighambardoust, Golshan Tafti, & Hesari, 2011; Chalat Santivarangkna, Kulozik, & Foerst, 2007). Water activity is also critical to the viability of dehydrated bacteria. It was observed that a water activity of lower than 0.25 was needed in order to maintain the viability of bacteria after spray drying (P. Teixeira, M. Castro, F. Malcata, & R. Kirby, 1995). It was also observed that a constant water activity was needed to improve the viability of spray-dried bacteria during prolonged storage (Chávez & Ledeboer, 2007).

### *5.4.7. Survivability of spray-dried L. salivarius during storage*

The viability of spray-dried *L. salivarius* during storage in a desiccator at 21 ºC is summarized in Tables 5-3-5-5. Overall, the viable cell counts in all spray-dried powder reduced gradually during 2-week storage. For RSM treatments produced at an outlet temperature of 94 ºC and above (Table 5-3), the reduction of viable bacteria was significant ($P < 0.05$) after one-week storage, while the reduction from the first week to the second week was insignificant ($P \geq 0.05$). Whereas, RSM treatments produced at an outlet temperature below 94 ºC showed significant ($P < 0.05$) reductions of viable bacteria after each week of storage. Similar trends were observed for

RSMST (Table 5-4) and RSMLT treatments (Table 5-5), with the exception of no significant reductions for the RSMLT treatment produced at an outlet temperature of 98-100 °C after two-week storage ($P \geq 0.05$). After two-week storage, the highest number of viable cells per unit mass of spray-dried powder (6.33 log CFU/g) was observed for the treatment spray-dried with RSMLT as the media, with prior heat adaptation, and at an outlet temperature of 84-86 °C.

Overall, powders prepared at a higher outlet temperature had a lower water activity (Table 5-2) that corresponded to better survivability of *L. saliarius* during storage in a desiccator (Tables 5- 3-5-5). As noted above, a water activity below 0.25 was recommended to maintain the viability of spray-dried bacteria during storage (P. Teixeira, M. Castro, F. Malcata, & R. Kirby, 1995). However, over-drying can also increase the mortality and instability of bacteria (de Valdez, De Giori, de Ruiz Holgado, & Oliver, 1985). Several studies reported the optimum survivability of bacteria during storage either at 4 °C or 20 °C of powders spray-dried to a water activity between 0.11 and 0.23 (Chávez & Ledeboer, 2007; Mary, Moschetto, & Tailliez, 1993; P. C. Teixeira, M. H. Castro, F. X. Malcata, & R. M. Kirby, 1995). Spray-dried samples in the present study had a water activity in the range of 0.109-0.246 (Table 5-2) but had different survivability during storage (Tables 5-3-5-5), which suggests the importance of media and stress adaption before spray drying. Bacteria spray-dried in media with disaccharides had a better survivability during storage than those of RSM. As discussed previously, disaccharides form hydrogen bonds to maintain the tertiary structures of proteins during drying (Leslie, Israeli,

Lighthart, Crowe, & Crowe, 1995) which may also have significance in maintaining the viability of bacteria during storage of powder in a desiccator.

### 5.5. Conclusions

Addition of sucrose, lactose and trehalose in RSM improved the viability of *L. salivarius* after spray drying. The mortality of spray-dried *L. salivarius* increased with the increase of outlet temperature. Heat adaptation of *L. salivarius* in RSMST and RSMLT additionally improved the bacterial viability after spray drying. Water activity of spray-dried *L. salivarius* increased with the decrease of outlet temperature and was lower than 0.25 in all samples. The lower water activity of powdered samples spray -dried from *L. salivarius* in RSMST and RSMLT corresponded to the improved survivability than RSM treatments after 2-week storage in a desiccator at 21 ºC. Overall, spray-drying *L. salivarius* in RSMLT with heat adaptation at an outlet temperature of 84-86 °C had the highest viable cell count after two-week storage. Therefore, the combination of the studied parameters can be used to produce powdered probiotic bacteria for functional foods applications.

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**Appendix**

Table 5-1. Log reductions after spray drying suspensions with ~9 log CFU/g solids *L. salivarius* NRRL B-30514 in RSM, RSMST or RSMLT, with and without prior heat adaption (HA) in the media, at an outlet temperature between 70 and 100 ºC.

| Outlet temperature (°C) | Reduction of *L. salivarius* *(log CFU/g) | | | | | |
|---|---|---|---|---|---|---|
| | RSM | | RSMST | | RSMLT | |
| | Without HA | With HA | Without HA | With HA | Without HA | With HA |
| 98-100 | $4.54\pm0.40^{a}$ | $3.91\pm0.38^{c}$ | $4.60\pm0.07^{a}$ | $3.43\pm0.01^{efgh}$ | $3.90\pm0.02^{c}$ | $3.32\pm0.02^{fghi}$ |
| 94-96 | $3.55\pm0.05^{defg}$ | $4.21\pm0.03^{b}$ | $3.82\pm0.04^{cd}$ | $3.00\pm0.02^{jkl}$ | $3.02\pm0.05^{jkl}$ | $2.82\pm0.10^{mn}$ |
| 90-92 | $3.28\pm0.03^{ghlj}$ | $3.20\pm0.02^{hijk}$ | $3.68\pm0.11^{cde}$ | $3.04\pm0.02^{ijkl}$ | $2.97\pm0.03^{kl}$ | $2.38\pm0.03^{nop}$ |
| 84-86 | $2.38\pm0.04^{nop}$ | $2.40\pm0.06^{nop}$ | $3.59\pm0.12^{def}$ | $1.69\pm0.07^{s}$ | $2.46\pm0.04^{no}$ | $1.17\pm0.03^{uv}$ |
| 80-82 | $2.32\pm0.05^{opq}$ | $2.29\pm0.04^{opqr}$ | $2.66\pm0.05^{mn}$ | $1.51\pm0.05^{st}$ | $2.04\pm0.05^{qr}$ | $1.13\pm0.06^{uv}$ |

Table 5-1. continued

| Outlet temperature (°C) | Reduction of *L. salivarius* *(log CFU/g) | | | | | |
|---|---|---|---|---|---|---|
| | RSM | | RSMST | | RSMLT | |
| | Without HA | With HA | Without HA | With HA | Without HA | With HA |
| 74-76 | 2.11±0.06$^{pqr}$ | 2.00±0.07$^{r}$ | 2.15±0.10$^{opqr}$ | 0.69±0.06$^{wx}$ | 1.65±0.03$^{st}$ | 0.90±0.06$^{vw}$ |
| 70-72 | 2.16±0.02$^{opqr}$ | 2.00±0.10$^{r}$ | 2.10±0.03$^{pqr}$ | 0.82±0.03$^{wx}$ | 1.38±0.12$^{tu}$ | 0.56±0.03$^{x}$ |

*Numbers are mean ± standard deviation from duplicate spray-dried samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 5-2. Water activity of powdered samples spray-dried from suspensions with ~9 log CFU/g solids *L. salivarius* NRRL B-30514 in RSM, RSMST or RSMLT, with and without prior heat adaption (HA) in the media, at an outlet temperature between 70 and 100 ºC.

| Outlet temperature (ºC) | Water activity* | | | | | |
|---|---|---|---|---|---|---|
| | RSM | | RSMST | | RSMLT | |
| | Without HA | With HA | Without HA | With HA | Without HA | With HA |
| 98-100 | $0.136\pm0.002^{rs}$ | $0.109\pm0.002^{u}$ | $0.141\pm0.002^{qr}$ | $0.137\pm0.002^{rs}$ | $0.120\pm0.004^{t}$ | $0.118\pm0.002^{t}$ |
| 94-96 | $0.153\pm0.003^{p}$ | $0.118\pm0.002^{t}$ | $0.138\pm0.002^{rs}$ | $0.171\pm0.002^{m}$ | $0.185\pm0.003^{ij}$ | $0.111\pm0.003^{u}$ |
| 90-92 | $0.182\pm0.002^{jk}$ | $0.157\pm0.003^{op}$ | $0.137\pm0.001^{rs}$ | $0.132\pm0.004^{s}$ | $0.170\pm0.003^{m}$ | $0.162\pm0.002^{no}$ |
| 84-86 | $0.189\pm0.004^{gh}$ | $0.180\pm0.004^{jk}$ | $0.160\pm0.004^{o}$ | $0.146\pm0.001^{q}$ | $0.185\pm0.003^{ij}$ | $0.123\pm0.001^{t}$ |
| 80-82 | $0.202\pm0.004^{g}$ | $0.177\pm0.004^{kl}$ | $0.167\pm0.004^{mn}$ | $0.159\pm0.004^{op}$ | $0.192\pm0.004^{g}$ | $0.161\pm0.001^{o}$ |
| 74-76 | $0.215\pm0.004^{f}$ | $0.231\pm0.004^{bc}$ | $0.189\pm0.003^{hi}$ | $0.170\pm0.002^{m}$ | $0.243\pm0.003^{a}$ | $0.173\pm0.004l^{m}$ |

Table 5-2. continued

| Outlet temperature (ºC) | Water activity* | | | | | |
|---|---|---|---|---|---|---|
| | RSM | | RSMST | | RSMLT | |
| | Without HA | With HA | Without HA | With HA | Without HA | With HA |
| 70-72 | 0.246±0.002[a] | 0.233±0.003[b] | 0.227±0.002[cd] | 0.222±0.002[de] | 0.245±0.003[a] | 0.221±0.002[de] |

*Numbers are mean ± standard deviation from duplicate spray-dried samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 5-3. Viable cell counts in powdered samples spray-dried from suspensions with ~9 log CFU/g solids *L. salivarius* NRRL B-30514 in RSM, with or without prior heat adaptation, at various outlet temperatures during 2-week storage in a desiccator at 21 ºC.

| Outlet temperature (°C) | Heat adaptation (HA) | Viable cell count (log CFU/g)* | | |
|---|---|---|---|---|
| | | Fresh | One week | Two weeks |
| 98-100 | Without HA | $4.68\pm0.40^{ij}$ | $4.15\pm0.34^{klm}$ | $4.05\pm0.21^{lmn}$ |
| | With HA | $5.31\pm0.38^{fg}$ | $4.44\pm0.46^{jk}$ | $4.40\pm0.21^{jkl}$ |
| 94-96 | Without HA | $5.01\pm0.03^{ghi}$ | $3.08\pm0.12^{r}$ | $3.21\pm0.21^{r}$ |
| | With HA | $6.10\pm0.04^{cd}$ | $3.64\pm0.34^{opq}$ | $3.55\pm0.05^{pqr}$ |
| 90-92 | Without HA | $5.92\pm0.07^{cde}$ | $3.96\pm0.21^{mno}$ | $3.29\pm0.16^{qr}$ |
| | With HA | $6.00\pm0.03^{cde}$ | $4.24\pm0.12^{klm}$ | $3.51\pm0.21^{pq}$ |
| 84-86 | Without HA | $6.84\pm0.03^{b}$ | $5.94\pm0.03^{cde}$ | $5.20\pm0.07^{gh}$ |
| | With HA | $6.82\pm0.06^{b}$ | $5.85\pm0.07^{cde}$ | $5.11\pm0.07^{gh}$ |
| 80-82 | Without HA | $6.90\pm0.05^{ab}$ | $6.06\pm0.16^{cd}$ | $4.85\pm0.05^{hi}$ |
| | With HA | $6..93\pm0.04^{ab}$ | $6.04\pm0.08^{cd}$ | $5.01\pm0.04^{ghi}$ |
| 74-76 | Without HA | $7.11\pm0.06^{ab}$ | $5.95\pm0.16^{cde}$ | $4.66\pm0.04^{ij}$ |
| | With HA | $7.22\pm0.07^{a}$ | $6.12\pm0.09^{c}$ | $5.05\pm0.02^{gh}$ |
| 70-72 | Without HA | $7.06\pm0.02^{ab}$ | $5.63\pm0.19^{ef}$ | $3.36\pm0.11^{qr}$ |
| | With HA | $7.22\pm0.10^{a}$ | $6.00\pm0.06^{cde}$ | $3.74\pm0.03^{nop}$ |

*Numbers are mean ± standard deviation from duplicate spray-dried samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 5-4. Viable cell counts in powdered samples spray-dried from suspensions with ~9 log CFU/g solids *L. salivarius* NRRL B-30514 in RSMST, with or without prior heat adaptation, at various outlet temperatures during 2-week storage in a desiccator at 21 ºC.

| Outlet temperature (°C) | Heat adaptation (HA) | Viable cell count (log CFU/g)* | | |
|---|---|---|---|---|
| | | Fresh | One week | Two weeks |
| 98-100 | Without HA | $5.32\pm0.07^{p}$ | $4.41\pm0.14^{u}$ | $4.17\pm0.11^{v}$ |
| | With HA | $5.57\pm0.03^{o}$ | $4.73\pm0.10^{t}$ | $4.82\pm0.11^{t}$ |
| 94-96 | Without HA | $6.10\pm0.04^{k}$ | $5.31\pm0.03^{p}$ | $5.30\pm0.08^{p}$ |
| | With HA | $6.01\pm0.02^{kl}$ | $5.30\pm0.05^{p}$ | $5.30\pm0.14^{p}$ |
| 90-92 | Without HA | $6.32\pm0.21^{j}$ | $5.76\pm0.07^{mn}$ | $5.21\pm0.03^{pq}$ |
| | With HA | $5.98\pm0.05^{kl}$ | $5.30\pm0.04^{p}$ | $4.87\pm0.04^{st}$ |
| 84-86 | Without HA | $6.33\pm0.12^{j}$ | $5.52\pm0.15^{no}$ | $5.08\pm0.07^{qr}$ |
| | With HA | $7.32\pm0.07^{e}$ | $6.99\pm0.07^{h}$ | $6.08\pm0.05^{k}$ |
| 80-82 | Without HA | $6.85\pm0.05^{i}$ | $6.40\pm0.03^{j}$ | $5.88\pm0.08^{lm}$ |
| | With HA | $7.19\pm0.05^{ef}$ | $7.12\pm0.03^{fg}$ | $6.00\pm0.01^{kl}$ |
| 74-76 | Without HA | $7.77\pm0.10^{bc}$ | $6.60\pm0.16^{i}$ | $5.62\pm0.11^{n}$ |
| | With HA | $8.32\pm0.06^{a}$ | $7.63\pm0.12^{cd}$ | $5.93\pm0.04^{klm}$ |
| 70-72 | Without HA | $7.82\pm0.03^{b}$ | $6.07\pm0.05^{k}$ | $5.01\pm0.05^{r}$ |
| | With HA | $8.19\pm0.03^{a}$ | $7.02\pm0.03^{h}$ | $5.36\pm0.05^{op}$ |

*Numbers are mean ± standard deviation from duplicate spray-dried samples and duplicate measurements for each sample. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 5-5. Viable cell counts in powdered samples spray-dried from suspensions with ~9 log CFU/g solids *L. salivarius* NRRL B-30514 in RSMLT, with or without prior heat adaptation, at various outlet temperatures during 2-week storage in a desiccator at 21 ºC.

| Outlet temperature (°C) | Heat adaptation (HA) | Viable cell count (log CFU/g)* | | |
|---|---|---|---|---|
| | | Fresh | One week | Two weeks |
| 98-100 | Without HA | $5.00\pm0.04^{opq}$ | $5.08\pm0.09^{nopq}$ | $4.92\pm0.12^{pqr}$ |
| | With HA | $5.01\pm0.05^{opq}$ | $5.06\pm0.03^{nopq}$ | $4.99\pm0.05^{pqr}$ |
| 94-96 | Without HA | $5.87\pm0.05^{hi}$ | $5.20\pm0.01^{lmn}$ | $5.11\pm0.02^{mno}$ |
| | With HA | $5.50\pm0.10^{kl}$ | $5.06\pm0.21^{nomq}$ | $5.03\pm0.06^{pqr}$ |
| 90-92 | Without HA | $5.89\pm0.07^{hi}$ | $5.66\pm0.07^{jk}$ | $5.09\pm0.06^{mnop}$ |
| | With HA | $5.92\pm0.06^{hi}$ | $5.29\pm0.03^{l}$ | $4.83\pm0.12^{r}$ |
| 84-86 | Without HA | $6.44\pm0.04^{f}$ | $5.70\pm0.02^{j}$ | $5.29\pm0.06^{l}$ |
| | With HA | $7.15\pm0.03^{c}$ | $6.72\pm0.05^{e}$ | $6.33\pm0.03^{f}$ |
| 80-82 | Without HA | $6.85\pm0.05^{de}$ | $6.15\pm0.08^{g}$ | $5.66\pm0.05^{jk}$ |
| | With HA | $7.19\pm0.05^{c}$ | $6.82\pm0.04^{de}$ | $5.79\pm0.06^{ij}$ |
| 74-76 | Without HA | $7.24\pm0.03^{c}$ | $6.38\pm0.11^{f}$ | $5.57\pm0.09^{jk}$ |
| | With HA | $7.42\pm0.06^{b}$ | $6.97\pm0.06^{d}$ | $6.04\pm0.01^{gh}$ |
| 70-72 | Without HA | $7.51\pm0.11^{b}$ | $5.98\pm0.01^{h}$ | $4.60\pm0.16^{rs}$ |
| | With HA | $7.76\pm0.03^{a}$ | $6.80\pm0.17^{de}$ | $5.25\pm0.02^{lm}$ |

*Numbers are mean ± standard deviation from duplicate spray-dried samples and duplicate measurements for each sample. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Figure 5-1. Survival of *L. salivarius* NRRL B-30514 after incubation at 45-55 ºC for up to 15 min in (A) RSM, (B) RSMST, and (C) RSMLT media. Error bars are standard deviations from duplicate independent samples, each sample measured twice.

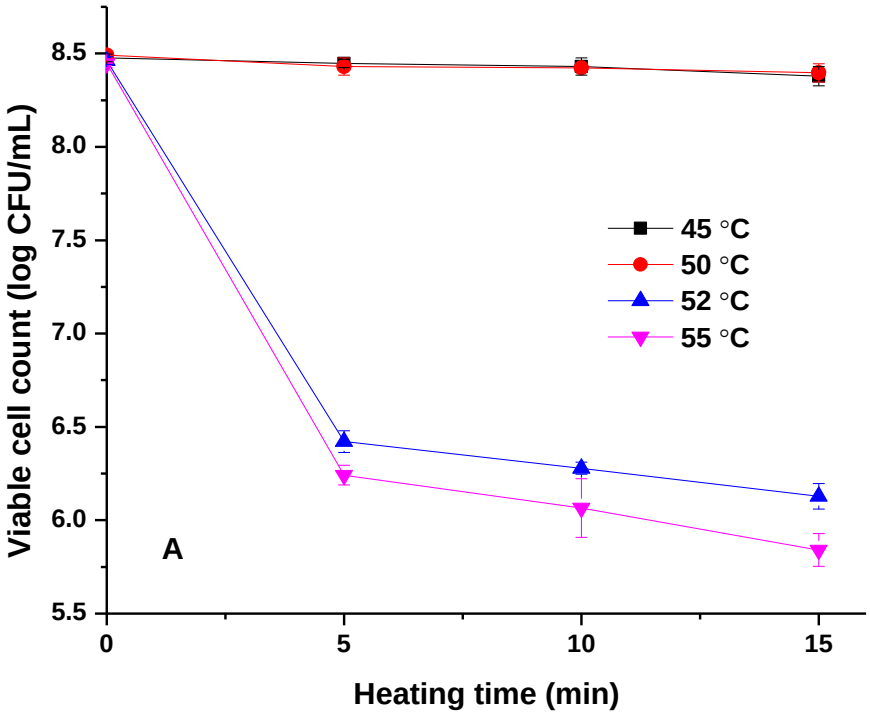


Figure 5-1. continued

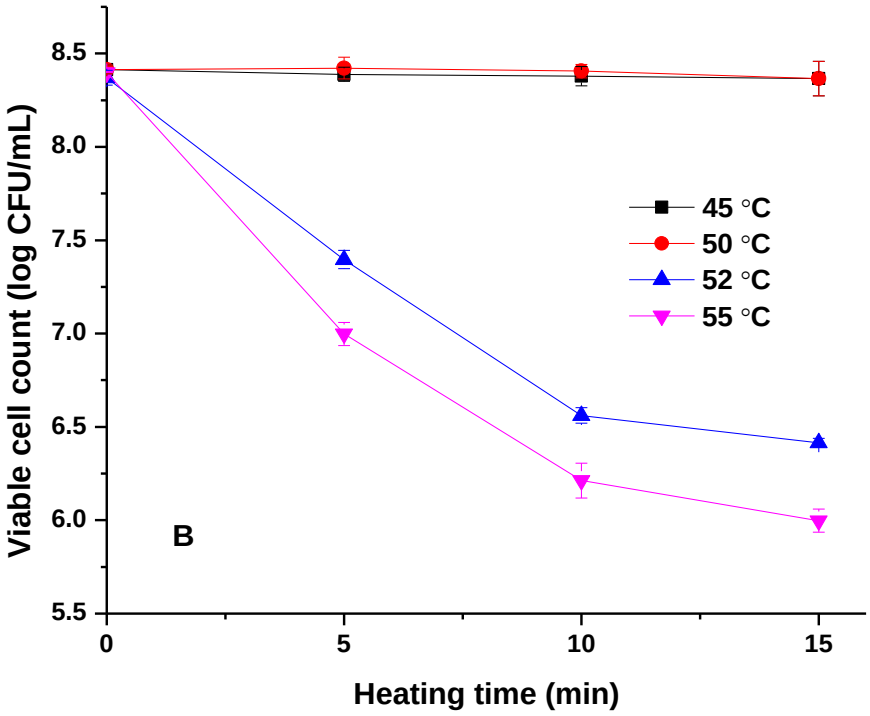


Figure 5-1. continued

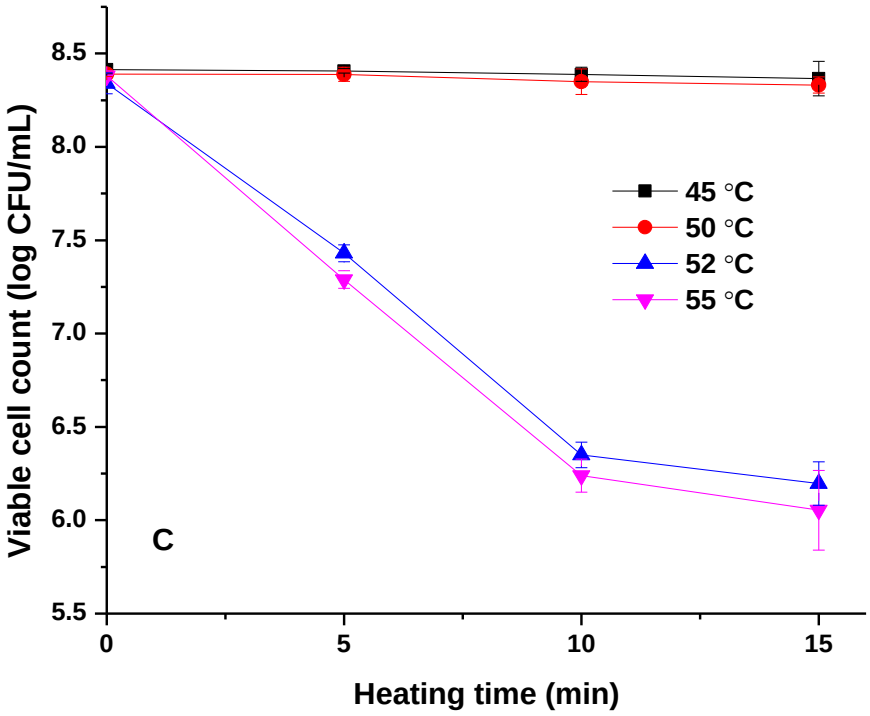


Figure 5-1. continued

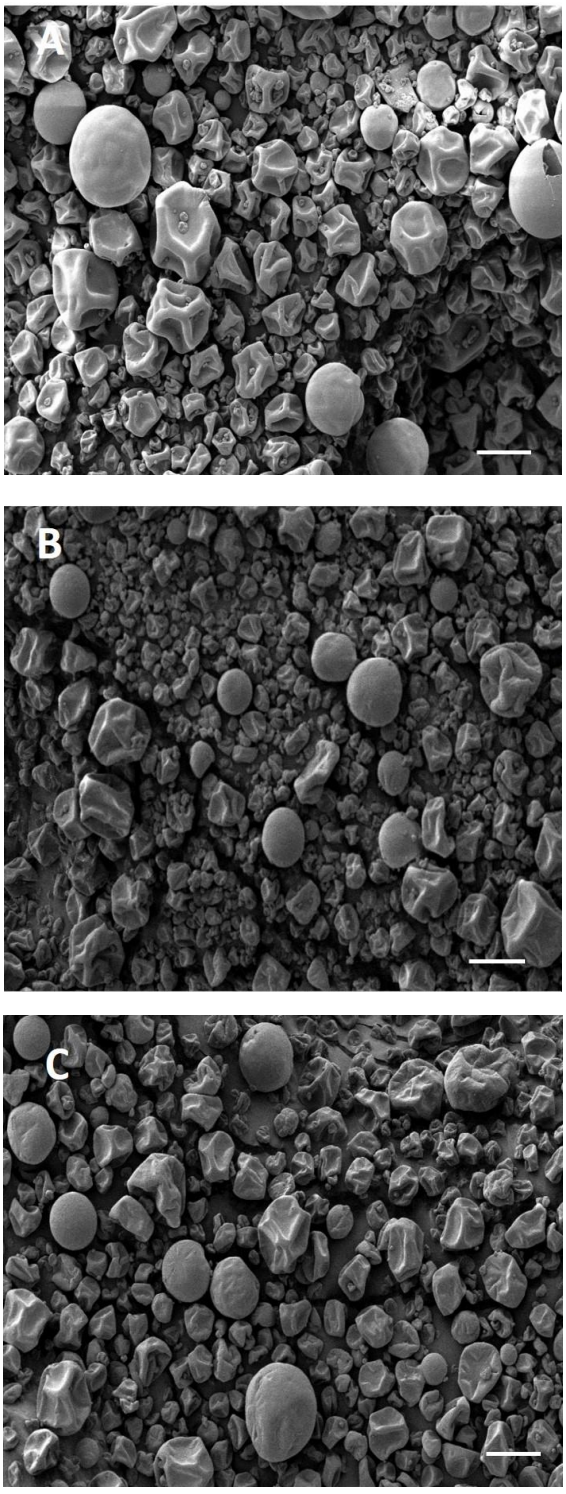


Figure 5-2. Scanning electron micrographs of powdered samples spray-dried from heat-adapted *L. salivarius* NRRL B-30514 suspended in (A) RSM, (B) RSMST, (C) RSMLT at an outlet temperature of 84-86 ºC. Scale bars equal to 20 µm.

# Chapter 6 . Effects of solid core composition on the viability of spray-dried *Lactobacillus salivarius* NRRL B-30514 encapsulated in S/O/W emulsions during storage and pasteurization

## 6.1. Abstract

Solid-in-oil-in water (S/O/W) emulsions can be used to encapsulate spray-dried probiotics as the solid core to improve the viability against environmental stresses. The objective of this work was to study effects of solid core composition, by spray-drying probiotic *Lactobacillus salivarius* NRRL B-30514 in different media to powders with different water activities, on the viability of probiotics encapsulated in S/O/W emulsions during storage at 4 ºC or 22 ºC and after pasteurization. Bacteria suspended in 20%w/v reconstituted skim milk (RSM), 18%w/v RSM+4%w/v sucrose+4%w/v trehalose, and 18%w/v RSM+4%w/v lactose+4%w/v trehalose were spray-dried at outlet temperature of 84-86 °C to a water activity range of 0.12-0.18 . Spray-dried *L. salivarius* suspended in soybean oil was homogenized at one-sixth volume in a 3% (w/v) of sugar beet pectin solution to prepare S/O/W emulsions. The encapsulation efficiencies were around 66%. Encapsulation reduced the mortality of *L. salivarius* during storage at 4 ºC or 22 ºC and pasteurization. Besides, spray-dried *L. salivarius* samples with a lower water activity and those prepared with disaccharides had a higher viability during storage at 22 ºC and a relative humidity of 11%. Therefore, the viability of probiotics encapsulated in S/O/W emulsions can be controlled by the composition of the solid core with spray-dried probiotics.

**Keywords:** spray-dried probiotics, S/O/W emulsion, water activity, drying media, viability

## 6.2. Introduction

The term probiotics was initially used by Lilly and Stillwell (Lilly & Stillwell, 1965). To date, probiotics have been defined in various ways, but the most common definition is "probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (R Fuller, 1989). Several health benefits of probiotics have been claimed, which involve positive effects on microflora balance (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010), immune system (Duerkop, Vaishnava, & Hooper, 2009), and intestinal discomfort (O'Mahony, McCarthy, Kelly, Hurley, Luo, Chen, et al., 2005). Due to these positive impacts on health, probiotics have been applied in pharmaceutical, food and poultry feed industries (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010; Patterson & Burkholder, 2003; Reuter, 2000). However, the viability of probiotic bacteria is affected by environmental factors such as pH, oxygen content, temperature, water activity, and digestive fluids (Rokka & Rantamäki, 2010). Practical applications of probiotics therefore require technologies that can decrease losses of viability and functionality of probiotics during processing and storage of products and before arriving at the target sites (Mattila-Sandholm, Myllärinen, Crittenden, Mogensen, Fondén, & Saarela, 2002). Such strategies have been studied for screening resistant bacteria strains, addition of protective substrates, stress adaptation, and encapsulation technologies (Champagne, Gardner, & Roy, 2005; Rokka & Rantamäki, 2010).

Emulsion is a widely investigated encapsulation technique to immobilize probiotic bacteria in order to preserve their viability during processing, storage and gastrointestinal tract (Rao, Shiwnarain, & Maharaj, 1989). The most significant advantage of emulsion technique is the small (<100 μm) dimension of capsules, which has less side effect on the texture of some food products (Chávarri, Marañón, Ares, Ibáñez, Marzo, & Villarán, 2010; Cook, Tzortzis,

Charalampopoulos, & Khutoryanskiy, 2012). General process for encapsulating probiotic bacteria by emulsions is to homogenize the mixture of bacteria polymer suspension and large volume of oil phase, after the water in oil emulsions is fabricated, water soluble polymer have to be cross linked to create beads within oil phase (Krasaekoopt, Bhandari, & Deeth, 2003). Various biopolymers are applied as supporting materials for encapsulating bacteria by emulsion technique, such as alginate (Poncelet, Poncelet De Smet, Beaulieu, Neufeld, & Goosen, 1993), chitosan (Zhou, Martins, Groboillot, Champagne, & Neufeld, 1998), carrageenan (Audet, Paquin, & Lacroix, 1988), and pectin (Guérin, Vuillemard, & Subirade, 2003). The size range of beads formed by emulsion technique is 25 μm to 2 mm (Krasaekoopt, Bhandari, & Deeth, 2003). Previously, we fabricated solid-in-oil-in-water (S/O/W) emulsions with sugar beet pectin (SBP) as a polymeric surfactant, spray-dried probiotic bacteria *Lactobacillus salivarius* NRRL B-30514 as the solid core, and soybean oil as the oil phase (Zhang, Lin, & Zhong, 2016). The S/O/W emulsions decreased the mortality of *L. salivarius* during storage, pasteurization, and *in vitro* digestion. Compared to the emulsion technique mentioned above, the S/O/W emulsion has a much smaller droplets dimension (less than 10 μm).

It is worthwhile to study effects of solid core composition on the survivability of spray-dried *L. salivarius* cells encapsulated in S/O/W emulsions because the viability of spray-dried probiotic bacteria during storage can be affected by drying media, drying temperature, prior stress adaptation, and water activity (Daemen & Van der Stege, 1982). In chapter 5, addition of dissacharides in reconstituted skim milk (RSM) improved the viability of *L. salivarius* after spray drying and during storage in a desiccator when compared to the RSM only treatment, and decreasing outlet temperature and application of heat adaptation also increased the survivability of spray-dried *L. salivarius*. For *L. salivarius* suspended in RSMST and RSMLT, spray-dried

samples with a lower water activity corresponded to better survivability after 2-week storage in a desiccator.

The objective of this study was to determine the survivability of *L. salivarius* spray dried in different media to various water activities after encapsulating in S/O/W emulsions. The S/O/W emulsions were prepared as previously reported (Zhang, Lin, & Zhong, 2016). The drying media were studied for RSM, RSM plus sucrose and trehalose (RSMST), and RSM plus lactose and trehalose (RSMLT). The viability of encapsulated *L. salivarius* was determined during refrigerator storage and after pasteurization of S/O/W emulsions. Additionally, the mortality of *L. salivarius* after spray-drying S/O/W emulsions was studied during ambient storage in a desiccator with the relative humidity (RH) of 11%.

## 6.3. Materials and methods

### *6.3.1. Materials*

SBP was a GENU® Explorer YA-400 product from CP Kelco (Atlanta, GA, USA). Soybean oil and pectinase from *Asperigillus niger* were purchased from MP Biomedicals, LLC. (Santa Ana, CA, USA). Organic nonfat dry milk powder was obtained from Organic Valley Family of Farms (La Farge, WI, USA). D-trehalose dihydrate (99%) was from Cascade Analytical Reagents and Biochemicals (Corvallis, OR, USA). Sucrose and D-lactose monohydrate were purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA). Other chemicals were from either Sigma-Aldrich Corp. (St. Louis, MO, USA) or Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA).

### *6.3.2. Preparation of spray-dried L. salivarius for encapsulation*

Spray-dried *L. salivarius* samples were prepared according to the conditions presented in our separate study. RSM, RSMST, and RSMLT were used as drying media. RSM is prepared by

suspending 20% w/v of non-fat milk powder in deionized water. RSMST was composed of 18% w/v non-fat dry milk, 4% w/v sucrose and 4% w/v trehalose. The composition of RSMLT was similar to RSMST, except the replacement of sucrose by lactose. Sterilization of these drying media was conducted in a 90 °C water bath for 30 min.

1 mL of frozen stock culture of *L. salivarius* was inoculated in 280 mL deMan, Rogoda and Sharpe (MRS) broth and incubated at 37 °C overnight in an anaerobic jar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cell pellets were harvested by centrifugation at 3000 *g* for 30 min using a centrifuge (RC-5B Plus, Sorvall, Inc. Norwalk, CT, USA) at 4 °C, and collected cells were washed twice using phosphate-buffered saline at pH 7.4 (PBS). The washed *L. salivarius* cells were added at 1% v/v in one of the three drying media mentioned above and incubated at 37 °C for about 5 h to obtain an exponential phase culture under an anaerobic environment, followed by heat adaptation in a 50 °C water bath for 15 min. After adaptation, the cell suspensions were spray dried using a BÜCHI B290 spray dryer (BÜCHI Corporation, Flawil, St. Gallen, Switzerland) at an inlet temperature of 170 °C and an outlet temperature of 84-86 °C. The spray-dried samples were denoted as free *L. salivarius* treatments. The water activity of these three samples was determined with an AquaLab Series 3TE instrument (Decagon Devices, Inc., Pullman, WA, USA).

### *6.3.3. Encapsulation of spray-dried L. salivarius in S/O/W emulsions*

Encapsulation of spray-dried *L. salivarius* was conducted busing a published method with some modification (Zhang, Lin, & Zhong, 2016). Briefly, spray-dried *L. salivarius* powder was mixed with soybean oil at 1:20 w/v on a stir plate to obtain a visually homogeneous S/O suspension. The aqueous solution was prepared by dissolving SBP at 3% w/v in deionized water

on stir plate overnight at room temperature , followed by centrifugation at 5,372 *g* for 20 min (model RC-5B Plus, Sorvall, Inc. Norwalk, CT, USA) to remove insoluble matter. The S/O suspension was homogenized at 12,000 rpm for 4 min in the SBP solution at an oil:aqueous phase volume ratio of 1:6 (IKA® 25 digital ULTRA TURRAX®, IKA® Works, Inc., Wilmington, NC, USA). Emulsions were re-adjusted to the SBP solution pH of 3.8.

### *6.3.4. Enumeration of L. salivarius*

Viable cell counts were enumerated using the pour plating method (Zhang, Lin, & Zhong, 2016). To enumerate viable cell counts of free bacteria, powder samples were suspended in the maximum recovery diluent (Thermo Scientific™ Oxoid™, Remel Inc, Lenexa, KS) at a concentration of 1 mg/mL. After serial dilutions, samples were pour plated on MRS agar. The plates were incubated at 37 °C for 48 h in anaerobic conditions before enumeration.

To enumerate viable cells encapsulated in S/O/W emulsions, pH value of emulsions was adjusted to 4.0 by 0.1 M NaOH, pectinase was added at a concentration of 0.5% w/v in emulsions, followed by incubation in a 25 °C water bath for 15 min to hydrolyze SBP to hydrolyze SBP on oil droplets. After hydrolysis of SBP, cells were collected by centrifugation at 15,280 *g* for 1 min (MiniSpin Plus centrifuge, Eppendorf, Inc. Hauppauge, NY, USA), and the collected cells were enumerated by the pour plating method presented above.

### *6.3.5. Dimension and zeta potential of droplets in S/O/W emulsions*

The dimension of droplets was measured using a model Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, UK). The arithmetic (number-averaged) mean diameter ($d_{1,0}$) of droplets was reported. Before measurement, emulsions were diluted 100 times using a 0.1 M phosphate buffer adjusted to pH 3.8.

The zeta potential of emulsion droplets was measured using the same Malvern instrument. Emulsions were centrifuged at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany) to remove free SBP. The precipitate was re-suspended in a phosphate buffer (pH 3.8, 0.1 M), and diluted 100 times using the same buffer before zeta potential measurement. The SBP solution at pH 3.8 was measured similarly.

### *6.3.6. Encapsulation efficiency (EE)*

EE was calculated as previously presented (Zhang, Lin, & Zhong, 2016). The encapsulated *L. salivarius* was released and enumerated ($C_e$) using the procedures in Section 6.2.4. The unencapsulated *L. salivarius* ($C_{free}$) was estimated by enumerating emulsions directly. The original live *L. salivarius* population used in encapsulation ($C_o$) was determined by centrifuging the S/O suspension, resuspending the precipitated cells in the same volume of PBS (pH 7.4), and enumerating as in section 6.3.4. EE was then calculated (Eq. 1).

$$EE\% = \frac{C_e(\text{CFU/mL}) - C_{free}(\text{CFU/mL})}{C_o(\text{CFU/mL})} \times 100 \quad (6\text{-}1)$$

### *6.3.7. Storage stability of L. salivarius encapsulated in S/O/W emulsions*

The S/O/W emulsions with encapsulated *L. salivarius* were stored in a refrigerator at 4 ℃. Suspensions hydrated with spray-dried free bacteria (0.1 g/mL) in 0.1 M PBS (pH 3.8) were used as controls. The viable cell counts of all samples were enumerated by the method in Section 6.3.4 after storage at 4 °C for 0, 7, and 14 days.

### *6.3.8. Viability of L. salivarius after pasteurization*

Suspensions as prepared in section 6.3.6 and S/O/W emulsions with encapsulated *L. salivarius* were heated in a water bath at 63 °C, and were held for 30 min after the sample center

reached 63 °C to simulate pasteurization conditions for full-fat milk (FDA, 2011). After cooling in a room-temperature water bath immediately, viable cell counts were determined using the methods in Section 6.3.4.

### *6.3.9. Viability of L. salivarius during storage of spray-dried capsules*

S/O/W emulsions with encapsulated *L. salivarius* were spray-dried using the above spray dryer at an inlet temperature of 170 °C and an outlet temperature of 84-86 °C. Spray-dried capsules were kept in glass vials with caps closed and stored in a desiccator at ambient temperature (22ºC) and an optimal relative humidity (RH, 11%) for preserving dried microorganisms (Castro, Teixeira, & Kirby, 1995). The relative humidity was created and maintained by a saturated lithium chloride solution (Greenspan, 1977). Spray-dried samples with free bacteria were studied as controls. After for 0, 7, and 14-day storage, spay-dried samples were suspended in the maximum recovery diluent, added with pectinase to hydrolyze SBP, and enumerated using the procedures detailed in Section 6.3.4.

### *6.3.10. Statistical analysis*

The mean and standard deviation of treatments were calculated from duplicate independent samples, each measured twice. One-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL).

## 6.4. Results and discussion

### *6.4.1. Characteristics of S/O/W emulsions with encapsulated L. salivarius*

Zeta potential, droplet dimension and encapsulation efficiency (EE) of S/O/W emulsions are summarized in Table 6-1. Zeta potential of the SBP solution at pH 3.8 was -69.73 mV, which was not significantly different ($P \geq 0.05$) from all emulsion samples after removing free SBP molecules. This indicates that the surface of oil droplets is mostly covered by SBP. Besides,

there was no significant difference ($P \geq 0.05$) among zeta potentials of three emulsion treatments. Zeta potential is important to the stability of colloidal systems, and emulsions prepared with polysaccharides with a zeta potential magnitude smaller than 30 mV are probably difficult to prevent flocculation and coagulation (Nakamura, Fujii, Tobe, Adachi, & Hirotsuka, 2012). The $d_{1,0}$ of droplets for all emulsions had no significant difference ($P \geq 0.05$). EEs of all three emulsions were all less than 70% and were not significantly different ($P \geq 0.05$).

### *6.4.2. Storage survivability of L. salivarius encapsulated in S/O/W emulsions*

The viable cell counts of *L. salivarius* in S/O/W emulsions during storage at 4 ℃ are shown in Table 6-2. Overall, viable cell counts decreased significantly for all samples after 14-day storage, and the reductions were more significant for free bacteria suspensions than those of emulsion samples. Specifically, there was about 5 log CFU/mL reduction for the viable cell counts of free bacteria suspensions, but the reductions were about 2.11, 1.85 and 1.46 log CFU/mL corresponding to emulsions with the solid core spray-dried with bacteria suspended in RSM, RSMST and RSMLT, respectively. This observation concurred with our previous study (Zhang, Lin, & Zhong, 2016). Besides, the reductions of viable cell counts in all emulsion samples had no significant difference ($P \geq 0.05$) after 7-day storage, but the reductions for RSM and RSMST emulsions stored for 14-day were significantly ($P<0.05$) higher than these stored for 7-day. It seems like the improvement of viability not only attributes to the isolation of bacteria from aqueous phase by oil droplets, but also the difference of water activity of spray-dried *L. salivarius* encapsulated in S/O/W emulsions. As mentioned above, free RSMLT had the lowest water activity.

Various studies claimed that encapsulation could improve the viability of probiotics during storage and under harsh conditions. For example, *Lactobacillus paracasei* ssp. *paracasei*

F19 and *Bifidobacterium lactis* Bb12 encapsulated in casein microcapsules formed by cross-linking with transglutaminase showed a protective effect on the viability of probiotic bacteria in a simulated gastric fluid (Heidebach, Först, & Kulozik, 2009). The survivability of *Lactobacillus plantarum* BL011 coated by sodium alginate or chitosan improved greatly under refrigerated storage compared to the free bacteria (Brinques & Ayub, 2011). *Lactobacillus rhamnosus* loaded in the inner water droplets of a water-in-oil-in-water emulsion prepared with sweet whey as an emulsifier showed the enhanced viability under low pH and bile salt conditions (Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter, 2009). Compared with these studies, the advantage of our study is that bacteria was isolated with water molecules by oil droplets, which could alleviate the mortality of bacteria.

### *6.4.3. Survival of L. salivarius encapsulated in S/O/W emulsions after simulated pasteurization*

Table 6-3 summarizes the viability of *L. salivarius* after heating at 63 °C for 30 min pasteurization. The viable cell counts for all free bacteria suspension samples after heating were under the detection limit, which agrees with the poor thermal tolerance of *L. salivarius* (Gardiner, O'sullivan, Kelly, Auty, Fitzgerald, Collins, et al., 2000). In contrast, encapsulation in S/O/W emulsions alleviated the mortality of probiotic cells after pasteurization. All emulsion treatments had about 1 log CFU/mL viable cells after heating, but the difference among emulsion treatments was not significantly different ($P \geq 0.05$). The improvement of bacterial viability after encapsulation as the solid core in emulsion droplets is probably because oil droplets can isolate bacteria with water molecules to diminish the mortality of bacteria during thermal treatment (Daemen, 1981).. Another possible explanation is that encapsulation creates a physical barrier to preserve the viability of bacteria under harsh conditions (Kailasapathy, 2002). Other studies also

reported that encapsulation could enhance the survivability of probiotic bacteria during heat stress. For example, eight strains of probiotic bacteria (*Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* type Bl-O4, and *B. lactis* type Bi-07) encapsulated in alginate beads had a better viability than free probiotics after heating at 65 °C for 30 min (Ding & Shah, 2007), for viable cell counts, encapsulated bacteria had an average loss of 4.17 log CFU/mL, 6.74 log CFU/mL loss for free bacteria. In a separate study, *Lactobacillus acidophilus* ATCC 43121 was encapsulated by dropping method using sodium alginate, the cell suspension was mixed with alginate solution and sprayed by an air-atomizing device (nozzle diameter is 0.4 mm) into $CaCl_2$ solution (0.5 mol/L) to form capsule, encapsulated and unencapsulated *L. acidophilus* ATCC 43121 were heated at encapsulated 65 °C for 30 min, viable cell counts of encapsulated *L. acidophilus* ATCC 43121 decreased from $1.2\times10^7$ to $2.1\times10^5$ CFU/ml, it reduced from $2.0\times10^7$ to $3.5\times10^4$ CFU/mL for unencapsulated ones (Kim, Cho, Kim, Song, Shin, Cha, et al., 2008). The reduction of viable cell in our study is higher than other studies mentioned above, which is probably due to the poor heat stability of *L. salivarius.*

### *6.4.4. Droplet size of S/O/W emulsions during storage and after simulated pasteurization*

The $d_{1,0}$ of S/O/W emulsions with encapsulated *L. salivarius* during storage at 4 °C and after heating at 63 °C for 30 min is shown in Table 6-4. Overall, the droplet dimension of all emulsions had no significant difference ($P \geq 0.05$) before and after 14-day storage at 4 °C, before and after heating. Besides, the appearance of emulsions had no difference visually before and after storage and heating. This result agrees with the conclusion obtained in section 6.3.1, S/O/W emulsions prepared with SBP had a good stability probably due to zeta potential. This result concurs with the good emulsifying property of SBP claimed by various studies (Drusch, 2007;

Nakauma, Funami, Noda, Ishihara, Al-Assaf, Nishinari, et al., 2008; Williams, Sayers, Viebke, Senan, Mazoyer, & Boulenguer, 2005).

### *6.4.5. Viability of spray-dried capsules during storage*

Table 6-5 shows the viability of spray-dried free and encapsulated *L. salivarius* during storage in a desiccator at 22 ºC and a RH of 11%. During spray drying, all spray-dried emulsion samples had a lower mortality than free bacteria samples (data not shown). Lethal thermal injury has been claimed as the major detrimental effect for the viability of bacteria during spray drying (Gardiner, et al., 2000; To & Etzel, 1997). Consequently, encapsulation provides a physical barrier for bacteria to maintain their viability during spray drying. In terms of subsequent storage, viable cell counts of all spray-dried emulsion samples were significantly lower ($P < 0.05$) than the corresponding free bacteria samples at day 0, but there was no significant difference ($P \geq 0.05$) after 14-day storage. This trend demonstrates that encapsulation improved the retention of viable cell counts of spray-dried probiotics during storage.

For free bacteria samples, the viable cell counts of all samples after 7-day were lower than that at day 0 ($P < 0.05$), and the differences of viable cell counts after 7-day and 14-day storage were also significant ($P < 0.05$). After 14-day storage, survivability of free bacteria spray-dried with RSM was significantly lower ($P < 0.05$) than those prepared with RSMST and RSMLT. This observation may be attributed by the positive effect of sucrose, lactose and trehalose on the viability of bacteria during spray drying and storage, because these disaccharides can stabilize membranes and proteins of bacteria during drying (Rudolph & Crowe, 1985). Additionally, this result also indicates that the water activity of spray-dried bacteria affects their survivability during storage. Water activity in either freeze or spray dried samples has been previously showed to influence the survivability of cells right after drying and during the

subsequent storage, lowest water activity could reduce the mortality of bacteria by minimizing water migration (Chávez & Ledeboer, 2007; Zayed & Roos, 2004). In this study, water activities of these three free bacteria samples were 0.18 for free RSM, 0.15 for free RSMST and 0.12 for free RSMLT, within the range of 0.11 and 0.23, which are considered as water activities can improve the survivability of dried samples during storage (Chávez & Ledeboer, 2007). However, the water activity of free RSMLT was the lowest, which may contribute to the highest viable cell counts of corresponding spray-dried emulsion sample. Like mentioned above, lower water activity results in a lower mortality of bacteria.

For spray-dried emulsion samples, viable cell counts had no significant change ($P \geq 0.05$) after 7-day storage for samples encapsulated with free RSMST and free RSMLT, the viable cell counts of sample encapsulated with free RSM were decreased significantly ($P<0.05$) after 7-day. For each treatment, the difference of viable cell counts after 7-day and 14-day storage was not significant ($P \geq 0.05$). This observation demonstrates that encapsulation enhances the survivability of spray-dried capsules during storage at 22 ºC and 11% RH. This is probably caused by the further reduced water activities of all spray-dried emulsion samples, their water activities were all around 0.13.

### 6.5. Conclusions

S/O/W emulsions were used to successfully encapsulate *L. salivarius* powder spray-dried from suspensions with different media to different water activities, and the EEs were about 68%. Encapsulation of *L. salivarius* in S/O/W emulsion reduced the mortality of probiotic bacteria during storage at 4 ºC and after the simulated pasteurization. Compared to free bacteria samples, further reduced water activity of spray-dried capsules could additionally enhance their viability

during storage at 22 ºC with RH of 11%. Drying media with disaccharides also had positive effect on the survivability of *L. salivarius* during storage at 22 ºC with RH of 11%.

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## Appendix

Table 6-1. Characteristics of S/O/W emulsions encapsulating spray-dried powder prepared from *L. salivarius* suspended in RSM, RSMST and RSMLT.*

| Medium used to prepare spray-dried powder | Zeta potential (mV) | Droplet size $d_{1,0}$ (μm) | EE (%) |
|---|---|---|---|
| RSM | -64.20 ± 2.75[a] | 7.97 ± 1.64[ab] | 66.64 ± 0.78[a] |
| RSMST | -63.93 ± 3.29[a] | 7.37 ± 0.73[abc] | 66.97 ± 1.72[a] |
| RSMLT | -66.60 ± 1.21[a] | 8.89 ± 0.64[a] | 68.85 ± 1.98[a] |

*Numbers are mean ± standard deviation from duplicate independent samples, each measured twice. Different superscript letters in the same column indicate significant differences in the mean ($P < 0.05$).

Table 6-2. Comparison of viability of *L. salivarius* suspended in a medium at pH 3.8 or encapsulated in emulsions as the solid core spray-dried from cells suspended in the same medium during storage at 4 ℃.

| Medium | Treatment | Viable cell count (log CFU/mL)* | | |
|---|---|---|---|---|
| | | 0-day | 7-day | 14-day |
| RSM | Free | $6.87\pm0.17^{ab}$ | $3.39\pm0.30^{h}$ | $2.15\pm0.04^{i}$ |
| | Emulsion | $6.00\pm0.32^{cd}$ | $5.18\pm0.20^{de}$ | $3.89\pm0.16^{fgh}$ |
| RSMST | Free | $7.09\pm0.73^{a}$ | $3.69\pm0.21^{gh}$ | $2.35\pm0.38^{i}$ |
| | Emulsion | $6.11\pm0.52^{bc}$ | $5.41\pm0.47^{cde}$ | $4.26\pm0.31^{fg}$ |
| RSMLT | Free | $7.16\pm0.79^{a}$ | $3.72\pm0.14^{gh}$ | $2.39\pm0.12^{i}$ |
| | Emulsion | $6.12\pm0.23^{bc}$ | $5.40\pm0.32^{cde}$ | $4.66\pm0.17^{ef}$ |

*Numbers are mean ± standard deviation from duplicate independent samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 6-3. Viability of free and encapsulated *L.salivarius* after pasteurization at 63 ℃ for 30 min.

| Medium | Treatment | Viable cell count (log CFU/mL)* | |
|---|---|---|---|
| | | Before heating | After heating |
| RSM | Free | $6.87\pm0.17^{ab}$ | <1 |
| | Emulsion | $6.00\pm0.32^{b}$ | $1.22\pm0.07^{c}$ |
| RSMST | Free | $7.09\pm0.73^{a}$ | <1 |
| | Emulsion | $6.11\pm0.52^{b}$ | $1.39\pm0.12^{c}$ |
| RSMLT | Free | $7.16\pm0.79^{a}$ | <1 |
| | Emulsion | $6.12\pm0.23^{b}$ | $1.36\pm0.11^{c}$ |

*Numbers are mean ± standard deviation from duplicate independent samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$). The detection limit is 1 log CFU/mL.

Table 6-4. Arithmetic mean diameter ($d_{1,0}$) of S/O/W emulsions with encapsulated spray-dried powder prepared from *L. salivarius* suspended in RSM, RSMST and RSMLT before and after storage at 4 °C and pasteurization at 63 °C for 30 min.

| Treatment | $d_{1,0}$ (μm)* | | |
|---|---|---|---|
| | RSM treatment | RSMST treatment | RSMLT treatment |
| Fresh | 7.97±1.64$^{ab}$ | 7.37±0.73$^{bc}$ | 8.89±0.64$^{a}$ |
| 7-day storage | 7.65±0.31$^{ab}$ | 6.64±1.31$^{bc}$ | 7.67±0.76$^{ab}$ |
| 14-day storage | 6.57±0.44$^{bc}$ | 6.08±0.52$^{c}$ | 7.34±0.46$^{bc}$ |
| Pasteurization | 6.76±0.44$^{bc}$ | 6.11±0.44$^{c}$ | 7.95±0.62$^{ab}$ |

*Numbers are mean ± standard deviation from duplicate independent samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

Table 6-5. Viability of powdered *L. salivarius* spray-dried from cells suspended in different media or corresponding emulsions during storage at 22 ºC and 11% RH.

| Medium | Treatment | Viable cell count (log CFU/g)* | | |
|---|---|---|---|---|
| | | 0-day | 7-day | 14-day |
| RSM | Free | $6.93\pm0.21^{bc}$ | $5.77\pm0.10^{fg}$ | $5.08\pm0.18^{i}$ |
| | Emulsion | $6.24\pm0.47^{def}$ | $5.62\pm0.11^{ghi}$ | $5.12\pm0.12^{hi}$ |
| RSMST | Free | $7.29\pm0.14^{ab}$ | $6.65\pm0.07^{cd}$ | $5.87\pm0.38^{fg}$ |
| | Emulsion | $6.51\pm0.56^{cde}$ | $6.02\pm0.08^{efg}$ | $5.66\pm0.26^{fgh}$ |
| RSMLT | Free | $7.59\pm0.16^{a}$ | $7.02\pm0.09^{bc}$ | $6.24\pm0.09^{def}$ |
| | Emulsion | $6.68\pm0.38^{cd}$ | $6.15\pm0.21^{defg}$ | $5.83\pm0.18^{fg}$ |

*Numbers are mean ± standard deviation from duplicate independent samples, each measured twice. Different superscript letters indicate significant differences in the mean of all samples ($P < 0.05$).

# Chapter 7 . Fabrication of S/O/W emulsions to encapsulate lactase for the retained enzymatic activity during dehydration, heating, and storage and the controlled release during *in vitro* digestion

## 7.1. Abstract

To supply milk products to lactose-intolerant consumers, lactose is hydrolyzed by lactase to produce lactose-free milk, but the adopted processes change quality parameters such as turbidity, viscosity, and flavors. The objective of this work was to study solid/oil/water (S/O/W) emulsions as delivery systems of lactase to retain the enzyme in milk and control the release of enzyme during in vitro digestion. Spray-dried lactase powders were prepared from four types of lactase products. To encapsulate lactase, spray-dried powder was suspended at 20% w/v in an oil phase with 90% w/w anhydrous milk fat and 10% w/w Span® 80 at 40 °C, while aqueous solutions with 5% w/v whey protein isolate or sodium caseinate and 1% w/v lecithin were prepared at neutral pH. The S/O suspension was emulsified into the aqueous protein solution at volume ratios of 1:6 and 1:8 to prepare S/O/W emulsions. The highest efficiency of encapsulating lactase in S/O/W emulsions was about 86%, and the corresponding emulsion had a hydrodynamic diameter of 357.3 nm and a zeta potential of -17.03 mV. Cross-linking the dialyzed emulsion with transglutaminase and addition of NaCas reduced free lactase from 41% to about 5% after spray drying emulsions. About 55% and 100% of activity was lost after heating free lactase at 60 and 72 ℃ for 30 min, while more than 60% of activity was retained for the encapsulated lactase. The hydrolysis of lactose in full-fat or skim milk after 14-day storage reduced from about 70% for free lactase to <20% for encapsulated lactase. The encapsulated lactase also was released gradually during the simulated digestions to hydrolyze lactose in milk. The present findings suggest S/O/W emulsions are potential delivery systems to incorporate lactase in milk products.

**Keywords**: lactase, encapsulation, S/O/W emulsions, controlled release, lactose hydrolysis

## 7.2. Introduction

Milk is an important source of essential amino acids, vitamins, minerals, energy, and fatty acids (Haug, Hostmark, & Harstad, 2007). Lactose is the most abundant solute (about 4.6% w/w) in milk and is digested in intestines by β-galactosidase (EC 3.2.1.23), commonly known as lactase (Vesa, Marteau, & Korpela, 2000). Lactose intolerance, which is caused by the deficiency of lactase in intestines, affects up to 100% of American Indians and Asians, showing symptoms such as bloating, abdominal pain and watery stool (Swagerty Jr, Walling, & Klein, 2002). In order to develop milk products suitable for lactose intolerant consumers, lactose is hydrolyzed by lactase to glucose and galactose to produce lactose-free milk (Dahlqvist, Mattiasson, & Mosbach, 1973; Scrimshaw & Murray, 1988). However, many lactose intolerant individuals dislike the sensory quality of lactose-free milk such as the increased sweetness (Onwulata, Rao, & Vankineni, 1989). Furthermore, the Maillard reaction during thermal deactivation of lactase can cause additional off-taste and browning (Harju, Kallioinen, & Tossavainen, 2012). Lactase is also available as an over-counter medicine to alleviate lactose intolerance symptoms (Swagerty Jr, Walling, & Klein, 2002), but its *in vivo* activity can be affected by proteases and pH in the gastrointestinal tract (Xenos, Kyroudis, Anagnostidis, & Papastathopoulos, 1998). Consequently, a novel solution may be to encapsulate lactase in particles that do not interfere texture, can retain the enzymatic activity during pasteurization and storage, and can control the enzyme release after ingestion to hydrolyze lactose *in vivo.*

Encapsulation of lactase has been studied previously. In a study (He, Zhang, & Sheng, 2014), lactase was encapsulated in a double-capsule delivery system composed of enteric-coated capsule and polylactic acid (PLA) nanocapsules (NCs). *In vitro* results showed that enteric-coated capsules remained intact in the simulated gastric fluid and protected the encapsulated

lactase from acidic denaturation. When the enteric coating collapsed rapidly and released lactase under the simulated intestinal conditions, the enzymatic activity and percentage of lactose hydrolysis (~100%) in milk were both much higher than the free lactase treatment (He, Zhang, & Sheng, 2014). In another study (Kwak, Ihm, & Ahn, 2001), medium-chain triacylglycerols and polyglycerol monostearate were used to microencapsulate lactase, coating material and lactase were mixed at 1200 rpm for 1 min with stirrer, followed by nebulizing the emulsion by an airless paint sprayer at 45°C into a cylinder containing a 0.05% Tween-60 solution at 5°C, the diameter of nozzle orifice was 0.4 mm, it has been concluded that a satisfied encapsulation efficiency (94.9 and 72.8%) was obtained, and encapsulated lactase had no negative effect on the taste of milk during 8 days storage at 5 °C. However, food-grade ingredients are to be used to deliver lactase in dairy products. In addition, the dimension of particles shall be small enough to prevent the sandy texture, which can be detected for particles as small as 10 μm in dairy products (Walstra, Walstra, Wouters, & Geurts, 2005).

Water-in-oil-in-water (W/O/W) emulsions are studied to deliver bioactive compounds, with the water soluble ones being dissolved in the inner water droplets (Garti & Bisperink, 1998). However, W/O/W emulsions have a low loading level, and the stability of inner water droplets and the deactivation of compounds during processing and storage are additional drawbacks (Couvreur, Blanco-Prieto, Puisieux, Roques, & Fattal, 1997; Vasiljevic, Parojcic, Primorac, & Vuleta, 2006). Solid-in-oil-in-water (S/O/W) emulsions can be studied to address the above challenges of delivering water-soluble compounds. For example, a study used poly(D,L-lactic-co-glycolic acid) and/or poly(D,L-lactic acid) to prepare S/O/W emulsions to encapsulate bovine superoxide dismutase and horseradish peroxidase efficiently without any loss of activity (Morita, Sakamura, Horikiri, Suzuki, & Yoshino, 2000). Insulin encapsulated in

S/O/W emulsions showed the improved absorption after oral administration (Toorisaka, Ono, Arimori, Kamiya, & Goto, 2003). These S/O/W emulsions however were not fabricated with food grade ingredients. In our previous studies, S/O/W emulsions were prepared with anhydrous milk fat (Poncelet, Poncelet De Smet, Beaulieu, Neufeld, & Goosen) as the oil phase, dairy proteins as surfactants, and spray-dried powder as the solid core. About 90% of glutamine was retained in S/O/W emulsions during 2-week storage at 5 °C (Zhang & Zhong, 2015). Encapsulation of probiotic *Lactobacillus salivarius* NRRL B-30514 in S/O/W emulsions improved the bacterial viability after spray drying and storage (Zhang, Lin, & Zhong, 2015). Therefore, S/O/W emulsions may be fabricated as delivery systems of lactase to achieve the above functions.

The first objective of the present study was to fabricate S/O/W emulsions with anhydrous MF as the oil phase and whey protein isolate (WPI) or sodium caseinate (NaCas) solution as the aqueous phase. To reduce S/O and O/W interfacial tensions, sorbitane monooleate 80 (Span® 80) and soy lecithin were dissolved in the oil and aqueous phases, respectively, to prepare emulsions. Four commercial lactase products were screened for the characteristics suitable for encapsulation. Emulsions were evaluated for the properties of preserving the activity of lactase during processing and storage. The second objective was to evaluate the release kinetics of lactase and the kinetics of hydrolyzing lactose in milk during *in vitro* digestion.

## 7.3. Materials and methods

### *7.3.1. Materials*

Five commercial lactase products were purchased from vendors and used without further purification: MP lactase from MP Biomedicals LLC. (Santa Ana, CA, USA), Ha-Lactase™ from Chr. Hansen, Inc. (Milwaukee, WI), Maxilact® LGi5000 (simplified as LGi5000 hereafter) and

Maxilact® LGX5000F (simplified as LGX5000F hereafter) from DSM Food Specialties (San Diego, CA), and ENZECO® fungal lactase from Enzyme Development Corporation (New York, NY). Span® 80 and Glucose (HK) Assay Kit was from Sigma-Aldrich Corp. (St. Luis, MO, USA). Soy lecithin (laboratory grade) was obtained from Fisher Scientific (Pittsburgh, PA, USA). WPI (93.4% protein, dry basis) was provided by Hilmar Ingredients (Hilmar, CA, USA). NaCas (93% protein, dry basis) was purchased from American Casein Co. (Burlington, NJ, USA). Anhydrous MF was provided by Land O'Lakes, Inc. (Arden Hills, MN, USA). The microbial transglutaminase (mTGase) (Activa TG-TI) was a product from Ajinomoto Food Ingredients, LLC (Chicago, IL). Skim milk and full fat milk were products of Horizon Organic (Broomfield, CO, USA). Other chemicals used in this study were obtained from either Sigma-Aldrich Corp. or Fisher Scientific.

### *7.3.2. Preparation of spray-dried lactase*

The liquid form lactase products (Ha-Lactase™, LGi5000, and LGX5000F) were spray-dried (model B290, BÜCHI Corporation, Flawil, St. Gallen, Switzerland) at a pump rate of 15%, an inlet temperature of 105 °C, and an outlet temperature of 56 °C. The MP lactase and ENZECO® fungal lactase products were in the powder form, but particles of ENZECO® fungal lactase were not spherical based on scanning electron microscopy. ENZECO® fungal lactase was dissolved in deionized water at 10% w/v and spray dried at the above conditions.

### *7.3.3. Characterization of powdered lactase*

*Protein electrophoresis*. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a precast 10% gradient polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA). Spray-dried lactase powder was dissolved at 1% w/v in water and diluted 10 times in an SDS–PAGE sample buffer with β-mercaptoethanol (Bio-Rad Laboratories

Inc., Hercules, CA, USA). After heating at 95 °C for 5 min, 10 μL of a sample was loaded onto the gel for electrophoresis at 200 V. The gel was stained by Coomassie brilliant blue G-250 and de-stained overnight. The Precision Plus Protein™ standard (Bio-Rad, Hercules, CA, USA) was used as a molecular weight marker.

*Powder morphology*. The lactase powder was imaged using a LEO 1525 SEM microscope (SEM/FIB Zeiss Auriga, Oberkochen, Germany). Before imaging, samples were glued onto an adhesive tape mounted on the specimen stub and sputter-coated with a gold layer of about 5 nm in thickness.

*Protein content*. Protein content of spray-dried lactase was measured with a bicinchoninic acid (BCA) method (Walker, 2009). Reagent A (Bio-world, Dublin, OH, USA) and Reagent B (prepared by dissolving 0.4 g $CuSO_4$ $5H_2O$ in 10 mL of deionized water) were mixed at a volume ratio of 50:1 to prepare the working reagent. Lactase were dissolved in water at a concentration of 10 mg/mL. After mixing a lactase sample with the working reagent and incubation at 60 °C for 30 min, samples were cooled to room temperature, and absorbance at 562 nm was measured to determine protein content based on a standard curve created using standard solutions of bovine serum albumin.

*Lactase activity*. Lactase activity was measured by a literature method (Jasewicz & Wasserman, 1961) with some modification. Spray-dried lactase was dissolved at 22.2, 28.6, and 40 mg/mL in 0.01 M phosphate-buffered saline (PBS) at pH 7.0, followed by mixing with 5.0% w/v lactose (in PBS) at a volume ratio of 1:2. The mixtures were incubated at 37 °C for 5 min, and the reaction was stopped by heating in a boiling water bath for 5 min. After cooling in an ice/water bath, the glucose content was determined using a glucose (HK) assay kit. One unit (U) of lactase is defined as the ability of enzyme to hydrolyze 1.0 μmol of lactose in one min.

*Thermal stability*. Lactase solutions were prepared from spray-dried powder at 40 mg/ml. The solutions were heated in a water bath at 72 °C for 15 s after sample center reached 72 °C to simulate thermal pasteurization of full fat milk (FDA, 2011). After heating, samples were cooled in an ice/water bath, and the activity of residual lactase was measured using the above method.

### *7.3.4. Encapsulation of spray-dried lactase particles in emulsions*

Based on results from preliminary experiments, spray-dried lactase powder was suspended at 20% w/v in an oil phase with 90% w/w MF and 10% w/w Span® 80 and blended using a Cyclone I.Q. microprocessor homogenizer (VirTis Co., Gardiner, NY, USA) to prepare a S/O suspension. Solutions with 5% WPI or NaCas were prepared at pH 7.0 and dissolved with 1% lecithin. The S/O suspension was emulsified into the protein solutions at volume ratios of 1:6 and 1:8 using the same homogenizer.

### *7.3.5. Encapsulation efficiency (EE)*

To measure EE according to Eq. 1, emulsions were centrifuged at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany), and the supernatant was collected to determine the activity of unencapsulated lactase using the glucose assay kit method in Section 7.3.3.

$$\text{EE\%} = \frac{Total\ lactase\ (\text{U}) - Unencapsulated\ lactase\ (\text{U})}{Total\ lactase\ (\text{U})} \times 100 \qquad (7\text{-}1)$$

### *7.3.6. Droplet size analysis*

The dimension of droplets was measured using a model Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, UK). The arithmetic (number-averaged) mean diameter ($d_{1,0}$) of droplets was reported.

### *7.3.7. Zeta-potential*

The zeta potential of emulsions was measured using the above Malvern instrument. Emulsions were centrifuged at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany) to remove free molecules. The precipitate was re-suspended in distilled water, adjusted to pH 7.0 using 1.0 M HCl or 1.0 M NaOH, and diluted 100 times using PBS (pH 7.0) before zeta potential measurement.

### *7.3.8. Spray drying of emulsions with encapsulated lactase*

Preliminary experiments indicated that a considerable amount of unencapsulated lactase was detected after spray drying S/O/W emulsions, especially for emulsions with LGi5000. Consequently, LGX5000F emulsions were studied for improvement of the amount of lactase remaining encapsulated after spray drying.

Emulsions were prepared by the same method mentioned above, except WPI and NaCas solutions were preheated at 80 ℃ for 15 min to facilitate cross-linking by mTGase (Wang, Zhong, & Hu, 2012). Emulsions were dialyzed overnight using a dialysis membrane with a molecular-weight-cut-off of 300 kDa to remove free molecules. The dialyzed samples were added with mTGase powder at a concentration of 10.2 U/mL in a 50 ℃ shaking water bath (New Brunswick Scientific Co., Edison, NJ, USA) for 4 h at pH 7.0 (Wang, Zhong, & Hu, 2012). After cross-linking, samples were spray dried at a pump rate of 15%. The inlet temperature was 120 ℃, and the outlet temperature was 55 ℃. To study effects of solids on spray-drying, another set of emulsions after cross-linking were supplemented with 5% (w/v) of NaCas, increased to pH 11.0 using 4.0 M NaOH and neutralized to pH 7.0 with 2.0 M HCl to facilitate the dissolution of caseins (Pan & Zhong, 2013), and spray-dried at identical conditions.

To evaluate the activity of lactase after spray drying, the spray-dried powder was suspended in deionized water at 1% w/v. Suspensions were centrifuged at 13,000*g* for 3 min to collect serum. As free lactase activity, the amount of lactase activity in serum was measured using the dispersion directly with the method in Section 2.3. To measure the total lactase activity in spray-dried powder, Tween 20 was added at 3.0% w/v into the suspension, and the mixture was kept in a refrigerator at 4 ℃ for about 20 h to displace proteins on emulsion droplets, followed by vortexing for 2 h at room temperature. After displacement, samples were centrifuged at 13,000*g* for 3 min to collect released lactase, lactase activity was measured by the method as above.

### *7.3.9. Thermal stability of lactase in dispersions hydrated with spray-dried capsules*

Spray-dried powders prepared with emulsions in Section 2.8 were rehydrated in distilled water on stir plate at room temperature for 1 h, and the suspension pH was adjusted to pH 7.0 using1.0 M HCl or NaOH. After heating in a water bath for 0.5, 1, 5, 10, 20 and 30 min after sample center reached 63, 72, or 90 ℃, samples were collected and cooled in an ice/water bath, and the residual lactase activity was measured by the methods in Section 7.3.8. Free lactase controls were studied for spray-dried powder as prepared in Section 7.3.2.

### *7.3.10. Hydrolysis of lactose in milk supplemented with lactase during refrigerated storage*

Spray-dried powders prepared in Section 7.3.9 or LGX5000F prepared in Section 7.3.2 were studied as representative encapsulated and free lactase for hydrolysis of lactose in milk during refrigerated storage. 0.03 g of lactase powder (about 20 Units) was suspended in 15 mL of skim or full fat milk on stir plate at room temperature for 1 h, followed by heating at 72 ℃ for 15 s to simulate pasteurization as above. The heated milk was stored in a refrigerator at 4 ℃. Samples were collected after storage for 0, 7 and 14 days. The collected samples were heated in

a 90 ℃ water bath for 10 min to inactive lactase, followed by adjusting pH to 4.6 to precipitate casein. After centrifugation at 13,000*g* for 5 min, the supernatant was used to measure the amount of hydrolyzed lactose by quantifying the molar amount of glucose using the HK glucose assay kit.

### *7.3.11. Release kinetics of lactase during in vitro digestion*

Simulated gastrointestinal fluids were prepared using the formula in published studies (Chávarri, Marañón, Ares, Ibáñez, Marzo, & Villarán, 2010; Oomen, Hack, Minekus, Zeijdner, Cornelis, Schoeters, et al., 2002). The simulated gastric fluid was composed of 3 g/L pepsin and 8.5 g/L sodium chloride, and the pH was 1.8. The simulated intestinal fluid contained 10 g/L pancreatin, 8.5 g/L sodium chloride, 3 g/L bile salt, and 10 g/L trypsin, and the pH was 6.5.

0.1 g of powder with free or encapsulated lactase as in Section 2.10 was rehydrated in 2 mL of distilled water, and the suspension was mixed with 15 mL of the simulated gastric fluid. Mixtures were incubated in a shaking water bath operating at 150 rpm and 37 ℃, and 1.0 mL aliquots were collected after 0, 1 and 2 h. The collected samples were adjusted to pH 7.0 with 0.1 M NaOH, followed by centrifugation at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany). The supernatant was used to determine lactase activity as in Section 7.3.3.

Mixtures after 2 h incubation in the simulated gastric fluid were adjusted to the composition simulating the intestinal fluid by dissolving bile salts, trypsin, and pancreatin directly to the aforementioned concentrations, and the pH was adjusted to 6.5 by 0.1 M NaOH. Samples were collected after 1, 2, 3, and 4 h incubation in the above shaking water bath to determine the amount of released lactase as described in the previous paragraph.

### *7.3.12. Hydrolysis of lactose in milk during in vitro digestion*

0.03 g of spray-dried samples (about 20 Units) were suspended in 2 mL of skim or full fat milk, followed by mixing with 15 mL of the simulated gastric fluid as prepared in Section 2.10. After incubation in the above shaking water bath at 37 ℃ for 0, 1, and 2 h, 1.0 mL samples were collected and centrifuged at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany), and the supernatants were used to measure glucose content using the HK glucose assay kit.

After 2 h incubation in the simulated gastric fluid, mixtures were adjusted to the simulated intestinal conditions as in Section 2.10=. During the subsequent 4 h incubation in the shaking water bath for 1, 2, 3, and 4 h, samples (1 mL) were collected, and adjusted to pH 4.6 by 0.1 M HCl to precipitate dairy proteins. After centrifugation at 13,000*g* for 3 min (Minispin plus centrifuge, Eppendorf, Hamburg, Germany), the supernatant was used to measure glucose content with the HK glucose assay kit.

### *7.3.13. Statistical analysis*

The mean and standard deviation were calculated from three independent replicates. One-way analysis of variance (ANOVA) was conducted using SSPS 16.0 (SSPS Inc., Chicago, IL).

## 7.4. Results and discussions

### *7.4.1. Purity and activity of lactase products*

The protein content and enzymatic activity of powdered lactase are summarized in Table 7-1. ENZECO® fungal lactase had the highest protein content, while the protein content of MP lactase was the lowest. There was no significant difference in the activity per unit mass of powder between spray-dried Ha-Lactase™, ENZECO® fungal, LGi5000, and LGX5000F samples ($P > 0.05$), while that of MP lactase powder was significantly lower ($P < 0.05$). When

calculated for specific activity (U/mg protein), MP, Ha-Lactase™ and LGX5000F powder had a similar specific activity ($P < 0.05$), while ENZECO® fungal lactase had the lowest specific activity. It appeared that the MP lactase contained a significant amount of non-protein matter and non-enzyme protein in the powder, i.e., the lowest purity.

SDS-PAGE analysis of lactase products is shown in Figure 7-1. The number of bands for MP was the least, which indicated the low protein content. The ENZECO® fungal, Ha-Lactase™, LGi5000 and LGX5000F had major bands between 150 and 250 kDa, as well as between 100 and 150 kDa. It was claimed that ENZECO® fungal, MP and Ha-Lactase™ were derived from *Aspergillus oryzae*, LGi5000 and LGX5000F were from dairy yeast *Kluyveromyces lactis*. However, it was concluded that *Aspergillus oryzae* β-galactosidase is a monomeric extracellular enzyme with a molecular weight of 90 kDa (Freitas, Marquez, Ribeiro, Brandão, Cardoso, & Ribeiro, 2011). Lactase from *K. lactis* were formed by two monomers of 124 kDa (Becerra, Cerdan, & Siso, 1998). The differences probably attribute to the different number and molecular mass of lactase's subunits, or different degree of glycosylation based on assay conditions (Tello-Solís, Jiménez-Guzmán, Sarabia-Leos, Gómez-Ruíz, Cruz-Guerrero, Rodríguez-Serrano, et al., 2005). Normally, lactase from fungi is applied to hydrolyze lactose in acidic products due to the most active pH range of 2.5-5.4 (Husain, 2010). Lactase from *Kluyveromyces lactis* is the most common lactase used to produce lactose-free milk because they possess the excellent lactose hydrolysis activity and an optimal pH range of 6.0-7.0 (Kim, Ji, & Oh, 2004).

### *7.4.2. Morphology of spray-dried powder*

Scanning electron micrographs of spray-dried lactase samples are shown in Figure 7-2. The MP lactase powder as received (Figure 7-2A) had mostly oval-shaped particles. The

dimension of spray-dried Ha-Lactase™ particles was around 20 μm (Figure 7-2B). The ENZECO® fungal lactase powder as received (Figure 7-2C) had sheet-like structures and became doughnut-like particles with a dimension around 10 μm after dissolving in water and spray-drying (Figure 7-2D). Spray-dried LGi5000 (Figure 7-2E) and LGX5000F (Figure 7-2F) samples had the smallest particles with a dimension smaller than 5 μm, which is desirable for preparing small capsules after encapsulation.

### *7.4.3. Stability of spray-dried lactase after simulated pasteurization*

The specific activity of solutions prepared from spray-dried lactase powder before and after the simulated pasteurization is shown in Table 7-2. The activity of MP, Ha-Lactase™, and ENZECO® fungal lactase decreased significantly ($P < 0.05$) after heating at 72ºC for 15 s, while the decrease was insignificant for LGi5000 and LGX5000F ($P > 0.05$). However, fungal lactase was thermostable but easy to be inhibited by produced galactose (Boon, Janssen, & Van‘t Riet, 2000). Considering particle dimension (Figure 7-2), heat stability (Table 7-2) and purity (Table 7-1), spray-dried LGi5000 and LGX5000F were chosen for encapsulation studies.

### *7.4.4. Particle size of emulsions*

The $d_{1,0}$ of S/O/W emulsions is shown in Table 7-3. Overall, emulsions prepared with a higher volume fraction of oil phase had bigger droplets, which probably is caused by the coalescence during emulsification due to the decreased amount of protein available to cover a larger surface area of oil droplets (Pandolfe, 1995). The $d_{1,0}$ of emulsions with LGX5000F was smaller than the comparable treatment with LGi5000 prepared with the same protein emulsifier and volume ratio of oil and aqueous phases, which can be explained by the smaller particles of spray dried LGX5000F (Figure 7-2).

### *7.4.5. Zeta potential of emulsions*

Zeta potentials of emulsions at pH 7.0 after removing free molecules are shown in Table 7-4. Zeta potentials of WPI and NaCas solutions were -19.47±1.50 mV and -20.30±0.72 mV, respectively. The zeta potential magnitude of emulsions after removing free molecules was generally smaller than that of the corresponding protein solution except for the LGi5000 treatment prepared with NaCas at a MF: protein solution volume ratio of 1:6.

### *7.4.6. Encapsulation efficiency (EE)*

The EEs of treatments are summarized in Table 7-5. Overall, the EE was higher at a lower volume fraction of MF, which corresponded to smaller droplets (Table 7-3). The treatments with WPI as the emulsifier and a MF: protein solution volume ratio of 1:8 resulted in a significantly higher EE than other treatments ($P < 0.05$), which was 85.59% and 76.33% for LGi5000 and LGX5000F, respectively. Consequently, the volume ratio of 1:8 was chosen to conduct further experiments.

### *7.4.7. Lactase activity of spray-dried capsules*

The activity of LGX500F lactase measured after rehydrating spray-dried samples is summarized in Table 7-6. Preliminary experiments showed that spray–dried emulsions without mTGase cross-linking treatment had about 30% of free lactase. mTGase treatment of dialyzed emulsions significantly decreased the portion of free lactase in spray-dried capsules ($P < 0.05$) which was further reduced after addition of casein for spray drying significantly. mTGase can cross-link dairy proteins (Wang, Zhong, & Hu, 2012), and this property has been utilized to improve the stability of emulsions prepared with proteins (Dickinson, 1997). The improved coalescence stability of oil-in-water emulsions prepared with sodium caseinate or β-lactoglobulin as an emulsifier was attribute to the increased elasticity of interfacial films after cross-linking by

mTGase (Dickinson, Ritzoulis, Yamamoto, & Logan, 1999; Færgemand, Otte, & Qvist, 1998). Another study showed the correlation between the higher polymerization degree of β-casein after mTGase treatment and the better storage stability of emulsions (Liu & Damodaran, 1999). Although the free lactase for spray-dried capsules with and without casein had no significant difference ($P$>0.05), addition of casein decreased the fraction of free lactase for all treatments. This may attribute to the creation of self-assembled casein particles (Pan & Zhong, 2013). Spray-dried capsules with mTGase treatment and addition of casein was used for further experiments.

### *7.4.8. Thermal stability of spray-dried capsules*

The thermal stability of lactase in dispersions hydrated with spray-dried capsules after heating at 63, 72 and 90 ℃ for 0.5, 1, 5, 10, 20 and 30 min is presented in Figure 7-3. Overall, the residual lactase activity of all treatments decreased to a greater extent after heating at a higher temperature for a longer duration. The residual activity of free lactase was less than 50% after heating at 63 ℃ for 30 min, which was significantly lower than the about 65% and 70% retention of lactase activity for emulsions prepared with WPI and NaCas, respectively. Free lactase was deactivated completely after heating at 72 ℃ and 90 ℃ for 5 min, but the encapsulated lactase had about 60% and 20% residual activity after heating at 72 ℃ and 90 ℃ for 30 min, respectively. Therefore, encapsulation improved the thermal stability of lactase. The treatment with the emulsion prepared with NaCas had a higher retention of lactase activity than the WPI treatment, which can be attributed to the better thermal stability of NaCas than WPI (McClements, 2004; Sliwinski, Lavrijsen, Vollenbroek, van der Stege, van Boekel, & Wouters, 2003).

### *7.4.9. Hydrolyzed of lactose in milk by free or encapsulated lactase during refrigerator storage*

The percentages of hydrolyzed lactose in full fat and skim milk hydrated with free or encapsulated lactase during 14-day storage at 4 ℃ are shown in Figure 7-4. For free lactase, about 65% lactose was hydrolyzed either in full fat milk or skim milk after 7-day storage, and the hydrolysis rate was much smaller during the next 7-day storage, reaching about 70% and 75% in full fat and skim milk, respectively. In comparison, the hydrolyzed lactose after 14-day storage was less than 15% and 20% in full fat milk and skim milk, respectively. The results showed the good stability of particles to retain the encapsulated lactase during refrigerated storage of milk.

### *7.4.10. Release kinetics of lactase during in vitro digestion*

The measured lactase activity during incubation of free and encapsulated lactase in simulated gastric and intestinal fluids is shown in Figure 7-5. During the first 2-h incubation in the simulated gastric fluid, the reduction of enzymatic activity of free lactase was about 95%, which is attribute to the degradation of protein caused by the pepsin and hydrochloric acid in simulated gastric fluid (Campbell, 2012). Besides, lactase from *K. lactis* are most active in pH of 6.0-7.0 (Genari, Passos, & Passos, 2003). For the dispersion hydrated with spray-dried capsules, about 10% of total initial lactase activity was released after the first 2 h incubation in the simulated gastric fluid, which reflected both the released lactase and the deactivated lactase. The release of lactase is likely caused by the demulsification after the protein layer on oil droplets is hydrolyzed by pepsin (Funami, Zhang, Hiroe, Noda, Nakauma, Asai, et al., 2007). Therefore, encapsulation of lactase in S/O/W emulsions reduced the loss of lactase activity during the simulated gastric digestion, which was also observed for lactase encapsulated in a double-capsule delivery system composed of enteric-coated capsules and polylactic acid nanocapsules (He, Zhang, & Sheng, 2014).

During the subsequent 4 h incubation in the simulated intestinal fluid, free lactase lost the activity completely in 2 h. This can be explained by the hydrolysis by proteases such as pancreatin and trypsin (Campbell, 2012; Zárate, Chaia, González, & Oliver, 2000). The steady increase of lactase activity for the encapsulated sample to about 50% of initial total activity indicates the rate of lactase release was higher than the deactivation rate by proteases. In addition to proteases hydrolyzing protein films on oil droplets, lipase from pancreatin can hydrolyze triacylglycerides of MF to accelerate the release of lactase (Bauer, Jakob, & Mosenthin, 2005; Favé, Coste, & Armand, 2004).

### *7.4.11. Hydrolysis of lactose in milk by free and encapsulated lactase during in vitro digestion*

Hydrolysis of lactose in full fat milk and skim milk during the simulated gastric (first 2 h) and intestinal (subsequent 4 h) digestions is summarized in Figure 6. In full fat milk (Figure 7-6A), the percentage of lactose hydrolyzed by free lactase group increased gradually during the first 2 h of the simulated gastric digestion, while the hydrolysis rate was much smaller in the subsequent 4 h incubation in the simulated intestinal fluid. This trend agreed with the stability of free lactase in the simulated gastric and intestinal fluids (Figure 7-5). In contrast, the lactose hydrolysis in treatments with encapsulated lactase showed was less than the free lactase treatment in the first 2 h, but the subsequent 4-h incubation in the simulated intestinal fluid resulted in a sustained increase of hydrolyzed lactose, corresponding to almost 70% of lactose hydrolysis at the end of incubation.

The trends of lactose hydrolysis in skim milk (Figure 7-6B) were very similar to those in full fat milk (Figure 6A). However, the percentages of hydrolyzed lactose in skim milk were slightly higher than the corresponding hydrolyzed lactose in full fat milk during 6 incubation. This phenomenon is probably caused by the difference of enzymatic activity of lactase in full fat

milk and skim milk, a study demonstrated that lactase activity was slightly higher in skim milk than in whole milk (Mahoney & Adamchuk, 1980). Therefore, lactase encapsulated in S/O/W emulsions is able to reduce the deactivation of lactase in the simulated gastric fluid to release most lactase for hydrolysis of lactose during the simulated intestinal digestion.

## 7.5. Conclusions

In conclusion, spray-dried lactase powder can be encapsulated in S/O/W emulsions with an encapsulation efficiency of up to 86%, but the type of lactase product differed in purity, thermal stability, and encapsulation efficiency. Dialysis of emulsions, treatment of emulsions with mTGase, and addition of Nacas reduced the amount of free lactase after spray drying Thermal stability of lactase in spray-dried capsules was improved significantly. Compared with free lactase, encapsulation of lactase reduced the hydrolysis of lactose in full-fat and skim milk during 2-week storage at 4 °C. Encapsulation preserved the enzymatic activity of lactase during the simulated gastric digestion and controlled the release of lactase during simulated gastric and intestinal digestions that improved the hydrolysis of lactose in full-fat and skim milk. These features of S/O/W emulsions show the potential of delivering lactase in milk.

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## Appendix

Table 7-1. Protein content, enzymatic and specific enzymatic activity of spray-dried lactase.

| Lactase product | Protein content (mg/g)* | Activity | |
|---|---|---|---|
| | | U/g powder | U/g protein |
| MP | 195.18±4.75$^{d}$ | 3392.83±59.73$^{b}$ | 17.38±0.12$^{a}$ |
| Ha-Lactase™ | 619.02±33.78$^{c}$ | 10828.97±44.13$^{a}$ | 16.78±0.81$^{a}$ |
| ENZECO® fungal | 989.96±15.21$^{a}$ | 10828.97±44.13$^{a}$ | 10.94±0.21$^{c}$ |
| LGi5000 | 856.94±16.02$^{b}$ | 10750.95±22.07$^{a}$ | 12.35±0.18$^{b}$ |
| LGX5000F | 643.76±11.93$^{c}$ | 10875.78±22.17$^{a}$ | 16.44±0.37$^{a}$ |

*Numbers are mean ± standard deviation (n = 3). Different superscript letters in the same parameter represent difference in the mean ($P < 0.05$).

Table 7-2. Specific activity of spray-dried lactase before and after at 72 ℃ for 15 s.

| Lactase product | Specific activity (U/g protein)* | |
|---|---|---|
| | Before heating | After heating |
| MP | 17.38±0.12[a] | 7.86±0.10[e] |
| Ha-Lactase™ | 16.78±0.81[a] | 8.38±0.05[d] |
| ENZECO® fungal | 10.94±0.21[c] | 4.21±0.11[f] |
| LGi5000 | 12.35±0.18[b] | 12.22±0.05[b] |
| LGX5000F | 16.44±0.37[a] | 16.36±0.03[a] |

*Numbers are mean ± standard deviation (n = 3). Different superscript letters represent difference in the mean ($P < 0.05$).

Table 7-3. The arithmetic mean diameter ($d_{1,0}$) of pH 7.0 S/O/W emulsions encapsulating spray-dried lactase particles with milk fat and protein solutions at 1:6 and 1:8 volume ratios.

| Protein emulsifier | Volume ratio of MF: protein solution | $d_{1,0}$ (nm)* | |
|---|---|---|---|
| | | LGi5000 | LGX5000F |
| WPI | 1:6 | 475.87±23.18$^{a}$ | 415.17±6.58$^{b}$ |
| | 1:8 | 433.23±12.89$^{b}$ | 357.27±16.39$^{c}$ |
| NaCas | 1:6 | 477.90±10.28$^{a}$ | 420.13±8.93$^{b}$ |
| | 1:8 | 313.43±4.92$^{de}$ | 291.90±1.78$^{f}$ |

*Numbers are mean ± standard deviation (n = 3). Different superscript letters represent difference in the mean ($P < 0.05$).

Table 7-4. Zeta potentials of pH 7.0 S/O/W emulsions encapsulating spray-dried lactase particles with milk fat and protein solutions at 1:6 and 1:8 volume ratios.

| Protein emulsifier | Volume ratio of MF: protein solution | Zeta potential* | |
|---|---|---|---|
| | | LGi5000 | LGX5000F |
| WPI | 1:6 | -15.07±0.31$^{h}$ | -18.63±1.37$^{bc}$ |
| | 1:8 | -15.73±0.21$^{fgh}$ | -17.03±0.31$^{def}$ |
| NaCas | 1:6 | -22.93±0.47$^{a}$ | -14.63±1.53$^{h}$ |
| | 1:8 | -18.30±0.44$^{cd}$ | -17.37±1.08$^{cde}$ |

*Numbers are mean ± standard deviation (n = 3). Different superscript letters represent difference in the mean ($P < 0.05$).

Table 7-5. Efficiencies of S/O/W emulsions encapsulating spray-dried lactase particles with milk fat and protein solutions at 1:6 and 1:8 volume ratios.

| Protein emulsifier | Volume ratio of MF: protein solution | Encapsulation efficiency (%)* | |
|---|---|---|---|
| | | LGi5000 | LGX5000F |
| WPI | 1:6 | $77.77 \pm 1.64^{bcd}$ | $73.58 \pm 0.46^{g}$ |
| | 1:8 | $85.59 \pm 0.26^{a}$ | $76.33 \pm 1.83^{cde}$ |
| NaCas | 1:6 | $62.02 \pm 0.78^{j}$ | $70.26 \pm 4.43^{hi}$ |
| | 1:8 | $67.93 \pm 0.43^{i}$ | $74.70 \pm 0.42^{efg}$ |

*Numbers are mean ± standard deviation (n = 3). Different superscript letters represent difference in the mean ($P < 0.05$).

Table 7-6. Lactase activity in suspensions hydrated with spray-dried capsules before (free) and after (total) adding Tween® 20 to release lactase. [#]

| Emulsion before spray drying | | Lactase activity (U/g powder)* | |
|---|---|---|---|
| Emulsifier | Addition | Free lactase | Total lactase |
| WPI | mTGase | 56.94±21.96$^{c}$ | 603.86±73.20$^{b}$ |
| WPI | mTGase + Casein | 16.82±5.50$^{c}$ | 617.23±5.49$^{b}$ |
| NaCas | mTGase | 51.76±24.40$^{c}$ | 741.88±24.40$^{a}$ |
| NaCas | mTGase + Casein | 33.64±7.32$^{c}$ | 635.34±1.83$^{b}$ |

[#] Emulsions encapsulating lactase LGX5000F powder were prepared with WPI or NaCas in the aqueous phase, dialyzed, added with mTGase to cross-link proteins on oil droplets, and spray-dried with or without adding 5% w/v NaCas.

*Numbers are mean ± standard deviation (n = 3). Different superscript letters represent difference in the mean ($P < 0.05$).

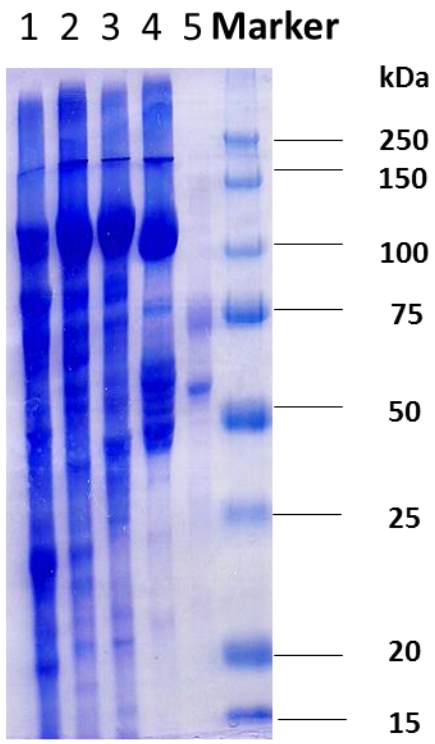



Figure 7-1. SDS-PAGE of analysis of LGX5000F (lane 1), LGi5000 (lane 2), Ha-Lactase™ (lane 3), ENZECO® fungal (lane 4), and MP (lane 5) lactase products.

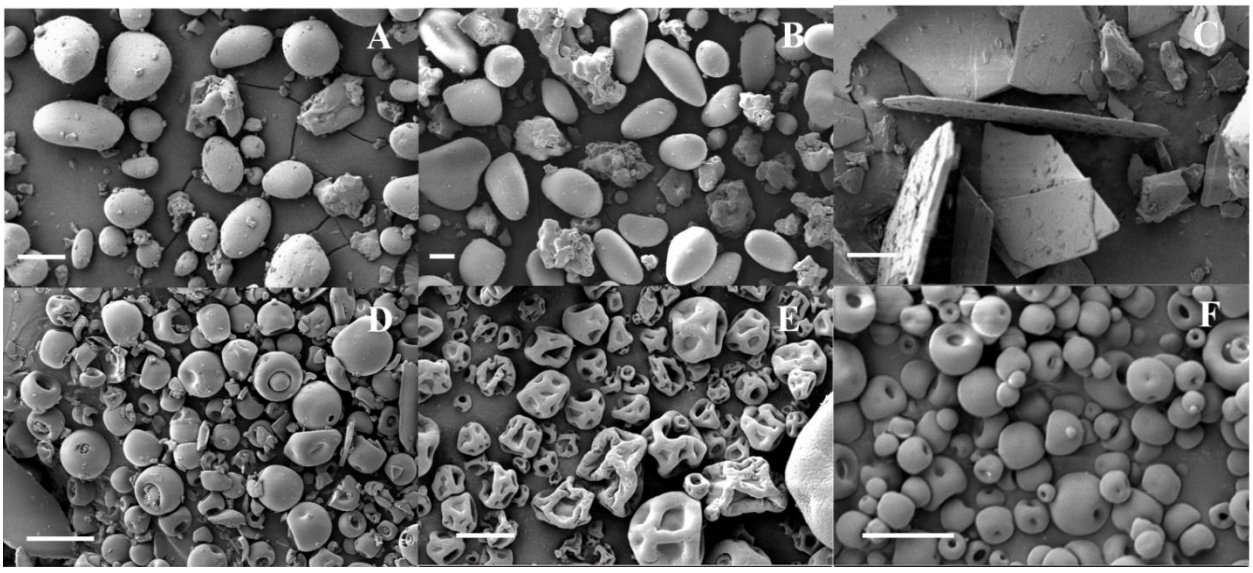
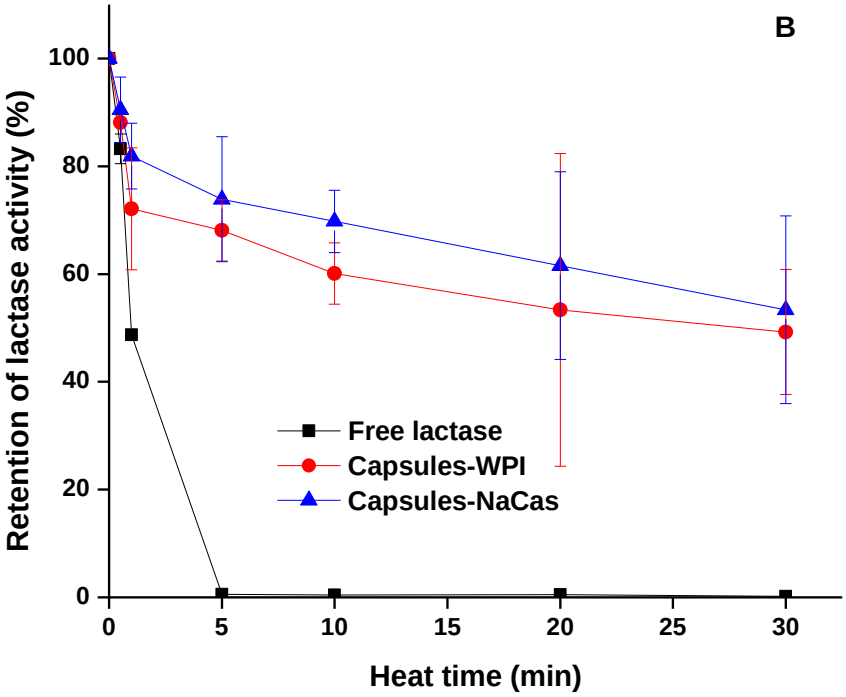



Figure 7-2. Scanning electron micrographs of (A) MP lactase as received, (B) spray-dried Ha-Lactase™, (C) ENZECO® FUNGAL LACTASE as received, (D) spray-dried ENZECO® FUNGAL LACTASE, (E) spray-dried 5000 lactase, and (F) spray-dried 5000F lactase. Scale bar = 20 μm in A-D, 5 μm in E and F.

Figure 7-3. Retention of lactase activity after heating neutral aqueous samples with free or encapsulated lactase at (A) 63 ℃, (B) 72 ℃, and (C) 90 ℃ for 0.5, 1, 5, 10, 20, and 30 min. The free lactase sample was prepared by hydrating spray-dried LGX5000F powder. The encapsulated lactase sample was prepared by hydrating spray-dried emulsions prepared with LGX5000F powder. Error bars are standard deviations from triple measurements.



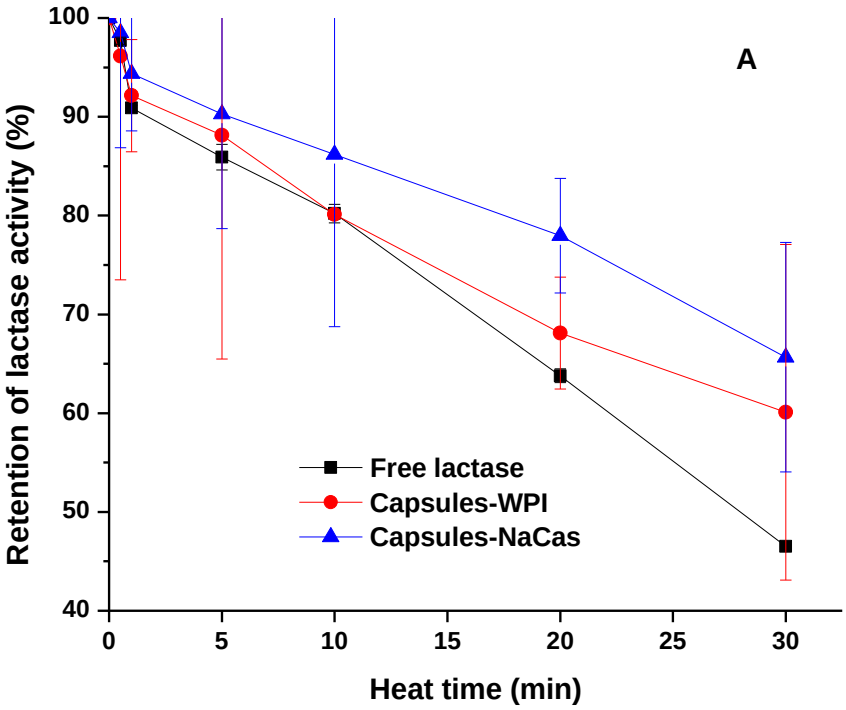




Figure 7-3. continued

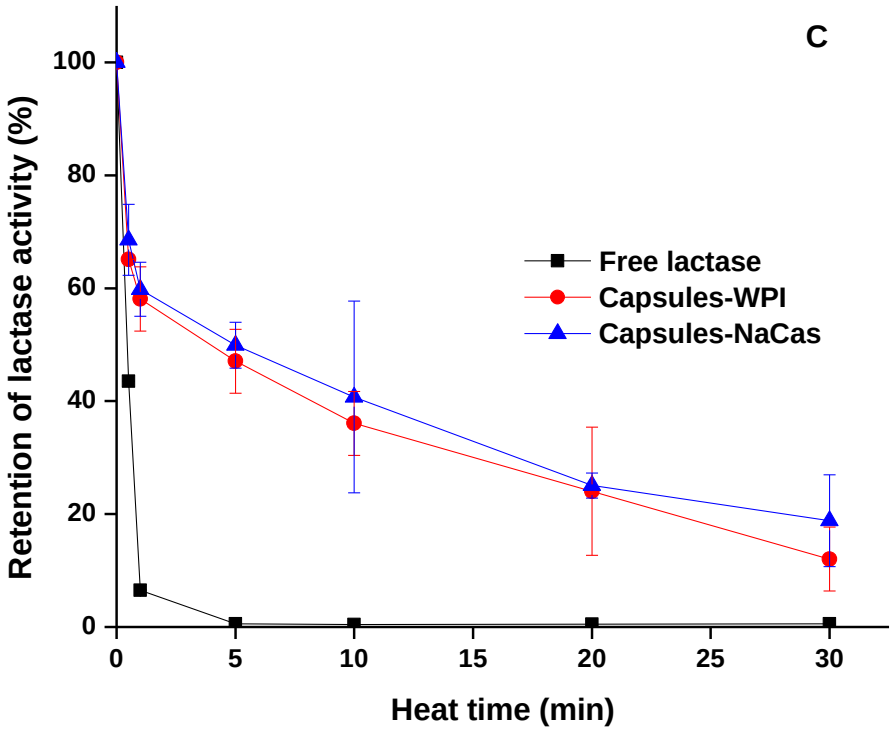



Figure 7-3. continued

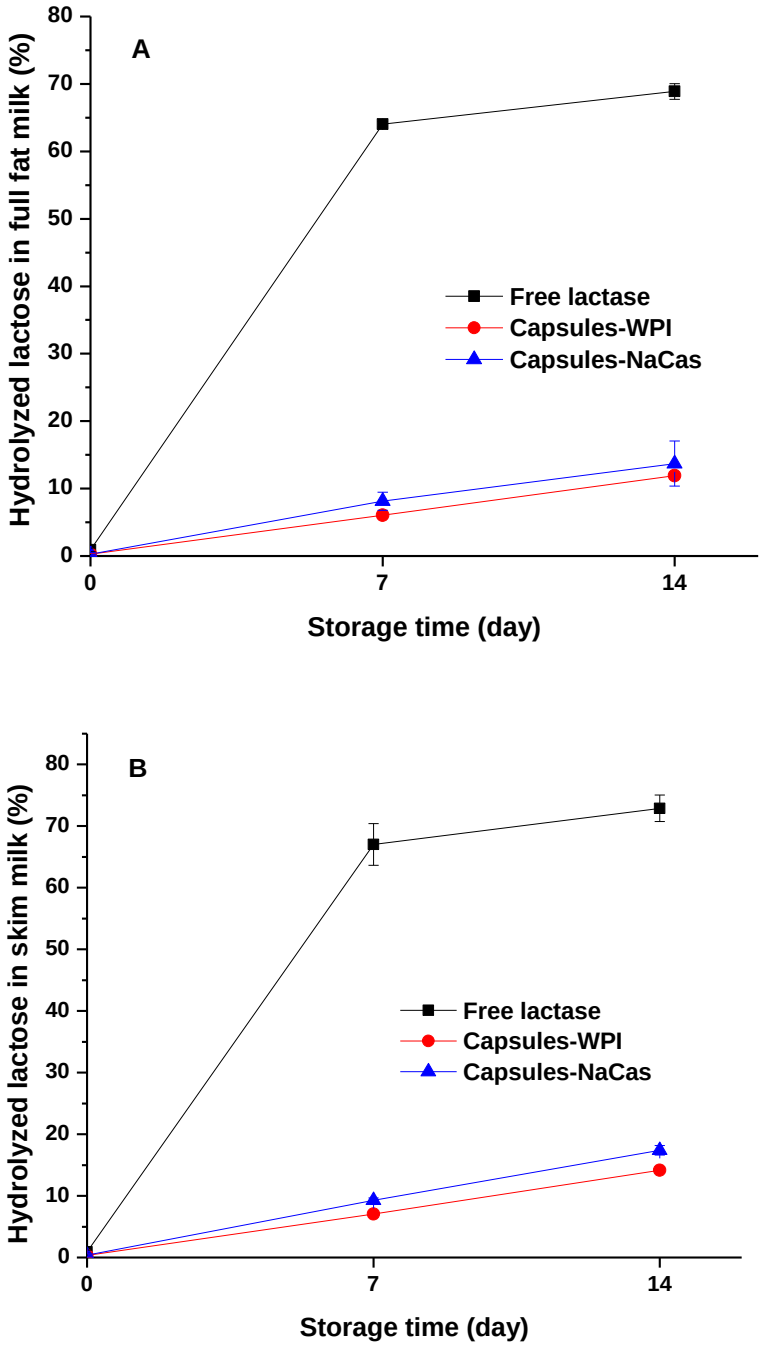



Figure 7-4. Percentages of lactose hydrolyzed in (A) full fat milk and (B) skim milk supplemented with free or encapsulated lactase during storage at 4 ℃ for 0, 7, and 14-day. Lactase samples are described in the caption of Figure 7-3. Error bars are standard deviations from triple measurements.

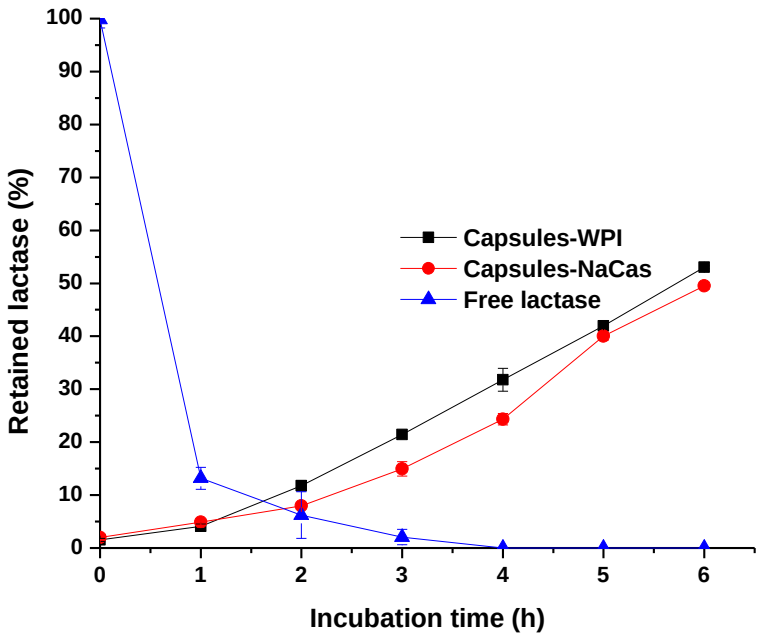



Figure 7-5. The measured lactase activity in simulated gastric (first 2 h) and intestinal (3-6 h) fluids added with free or encapsulated lactase prepared with WPI or NaCas. Lactase samples are described in the caption of Figure 7-3. Error bars are standard deviations from triple measurements.

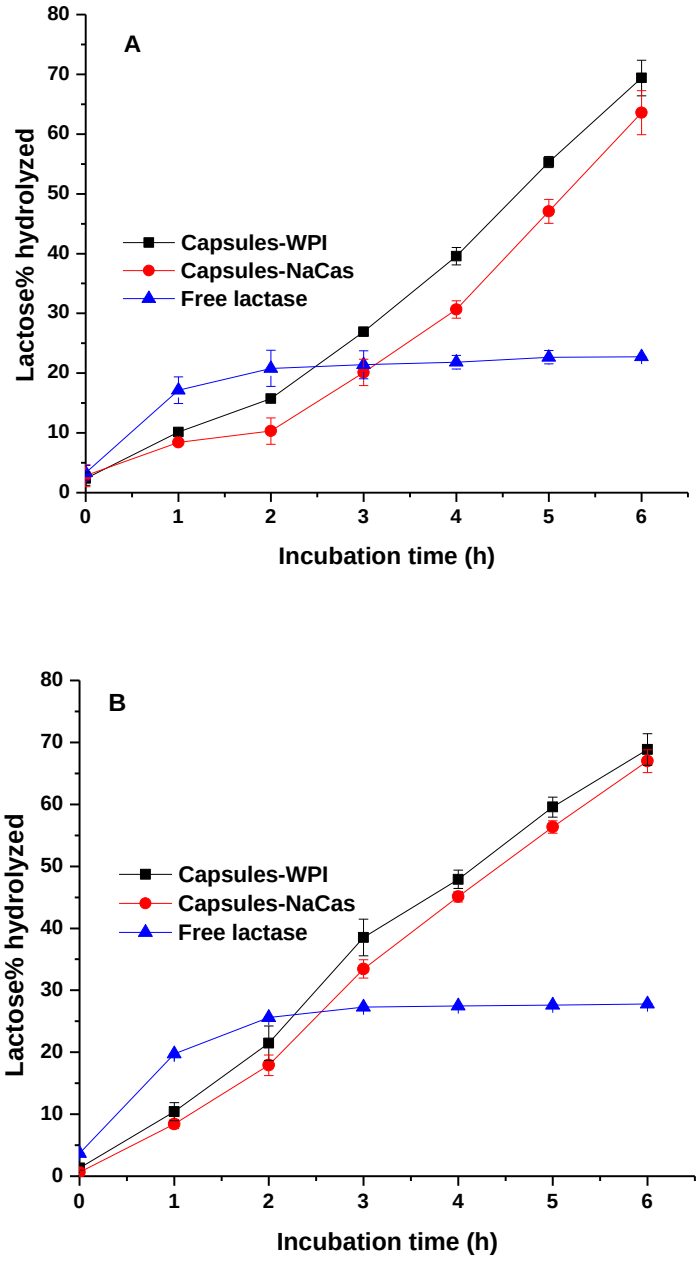



Figure 7-6. Hydrolysis of lactose in full fat milk (A) and skim milk (B) supplemented with free or encapsulated lactase during simulated gastric (first 2 h) and intestinal (3-6 h) digestions. Lactase samples are described in the caption of Figure 7-3. Error bars are standard deviations from triple measurements.

# Chapter 8 . Concluding remarks and future work

## Conclusions

This dissertation demonstrated that colloid delivery systems fabricated by milk proteins and polysaccharides (pectin and sugar beet pectin) preserved the activity of bioactive food ingredients, such as glutamine, probiotic bacteria and lactase, during processing, storage, and simulated gastrointestinal conditions.

Multiple-layered emulsions prepared with WPI or NaCas and pectin can encapsulate glutamine solid particles. After encapsulation, water-soluble glutamine had a higher retention during the refrigerated storage. All treatments showed >90% release after the subsequent incubation for 4 h in the simulated intestinal fluid.

*L. salivarius* can be encapsulated multiple-layered emulsions with an EE of up to 90%. The encapsulation technology as proposed resulted in microcapsules that have promise for food applications without causing sandy texture. The encapsulation improved the storage and thermal stability of *L. salivarius*. The encapsulated *L. salivarius* also had the significantly enhanced stability after spray drying. Lastly, encapsulation of the probiotic cells in the secondary emulsion, particularly after cross-linking by calcium ions, greatly improved the survivability during the simulated gastric and intestinal digestions.

SBP can be used to prepare S/O/W emulsions to encapsulate *L. salivarius* with an EE of up to about 87% and droplets smaller than 17 μm. Encapsulation of *L. salivarius* in S/O/W emulsions improved the viability during storage, pasteurization, and *in vitro* digestions. Cross-linking SBP on emulsion droplets by divalent calcium ions additionally improved the viability of *L. salivarius* against various environmental stresses deactivating the bacterium, enabling the survival of a significant portion of viable *L. salivarius* after simulated gastrointestinal digestions.

RSM with sucrose, lactose and trehalose improved the viability of *L. salivarius* after spray drying. The viability of spray-dried *L. salivarius* increased with the decrease of outlet temperature. Heat adaptation of *L. salivarius* in RSMST and RSMLT additionally improved the bacterial viability after spray drying. Water activity of spray-dried *L. salivarius* increased with the decrease of outlet temperature and was lower than 0.25 in all samples. Spray-drying *L. salivarius* in RSMLT with heat adaptation at an outlet temperature of 84-86 °C had the highest viable cell count after two-week storage. Therefore, the combination of the studied parameters can be used to produce powdered probiotic bacteria for functional foods applications.

S/O/W emulsions prepared with SBP encapsulated spray-dried *L. salivarius* with different water activities and drying media successfully, and the EEs were about 68%. Encapsulation of *L. salivarius* by S/O/W emulsion can diminish the mortality of probiotic bacteria during storage at 4 ºC or 22 ºC and pasteurization. For spray-dried capsules, lower water activity of spray-dried *L. salivarius* samples could enhance the viability of bacteria during storage at 22 ºC with RH of 11%. Drying media with disaccharides for *L. salivarius* also had positive effect on the survivability of bacteria during storage at 22 ºC with RH of 11%.

S/O/W emulsions prepared with WPI or NaCas encapsulated spray-dried lactase with an EE of up to 86%. Incorporation of transglutaminase (mTGase) and casein, demulsification was alleviated during spray drying, which decreased the free lactase of spray-dried capsules. Thermal stability of spray-dried capsules was improved significantly. Encapsulation of lactase retarded the hydrolysis of lactose in either full-fat or skim milk induced by lactase during storage at 4 °C. Encapsulation preserved the enzymatic activity of lactase and controlled the release of lactase during simulated gastric and intestinal digestions. With the improved enzymatic activity of

lactase by encapsulation, hydrolysis of lactose in full-fat and skim milk was increased dramatically during simulated gastric and intestinal digestions.

**Future work**

Results presented in this dissertation conclude that colloidal delivery systems can be fabricated to preserve the activity of bioactive food ingredients, as well as to control the release of these ingredients in simulated gastrointestinal conditions. However, future *in vivo* studies are needed to verify the studied emulsions as a potential system to deliver compounds in foods to improve their activity during processing, storage, and digestion. Specifically, *in vivo* studies can be conducted to measure the cell counts of encapsulated probiotic bacteria after ingestion and passing through the gastrointestinal tract. Besides, *in vivo* studies can also tract the effect of encapsulated lactase in milk products on lactose intolerance.

# VITA

Yun Zhang was born in a small town in China on November 3rd, 1987. After graduating from high school, she went to Nanjing Agricultural University to pursue a bachelor degree in Food Quality and Safety. She became a master graduate without examination in Food Science and Technology department of Nanjing Agricultural University in 2010. Later on, she begun to apply PhD degree in United States, and obtained an offer from Dr. Qinxin Zhong luckily. As a PhD student, she enrolled in University of Tennessee, Knoxville in 2012. Until now, her life is moving forward step by step according to her thought. She has no idea about her future life, but she will keep moving with her primal belief.